**PATENT**
Attorney Docket No. 225011

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

Flack et al.

Group Art Unit: 1614

Application No. 10/806,088

Examiner: James D. Anderson

Filed: March 3, 2004

For: GOSSYPOL FOR THE TREATMENT OF CANCER

**DECLARATION UNDER 37 C.F.R. § 1.132 OF MARCUS REIDENBERG**

I, Marcus Reidenberg, hereby declare the following:

1. I am the a co-inventor of the subject matter that is disclosed and claimed in the matter of the above-captioned application, and I have considerable experience in the field of the present invention as evidenced by the attached copy of my *curriculum vitae* (Exhibit A).

2. I am familiar with the application and the pending claims.

3. I am a coauthor of "Oral gossypol in the treatment of patients with refractory metastatic breast cancer: A phase I/II clinical trial," Van Poznak et al., *Breast Cancer Res. and Treatment*, 66: 239-48 (2001).

4. The study reported in the Van Poznak article was conducted with cancer patients for whom there were no other standard treatment options. All participants had metastatic cancer and had experienced at least two previous regimens of chemotherapy treatment. It was not expected that the participants in this particular study, all of whom were

in very advanced stages of cancer with limited life expectancy, would experience an improvement due to treatment with gossypol despite our hope that improvement might occur in some. The study was conducted primarily to determine what dose(s), achievable in patients, would lead to a change in pRb and cyclin D1 protein expression. This was a follow up to our previous cell study described in paragraph 7.

5. To characterize the Van Poznak study as demonstrating that the treatment of cancer with gossypol is unpredictable is, in my opinion, erroneous. At most, the study shows that gossypol exhibited some effects in a small population of heavily pretreated patients with metastatic breast cancer, for whom there were no other standard treatment options. The study also showed that a range of plasma concentrations were safe and well tolerated and the article concludes that gossypol "may be considered a lead compound for a new class of antineoplastic agents."

6. Recent findings support the expectation that (-)-gossypol is useful for the treatment of a wide diversity of cancers in humans. It has been reported that modulating apoptosis (programmed cell death) suppressing members of the Bcl-2 family holds potential for treating cancer. See Shore et al., *J. American Society of Hematology*, 226-230 (2005) (Exhibit B). Apoptosis suppressors (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1) are often strongly elevated in diverse cancers, and have potential to confer resistance to endogenous cell death stimuli and many cancer treatments. Thus, a small organic molecule that binds multiple Bcl-2 family members is expected to be useful for the treatment of diverse cancer cell types. (-)-Gossypol binds to Bcl-2, Bcl-xL, and Mcl-1 proteins, with high affinity. As (-)-gossypol binds three members of the Bcl-2 family, (-)-gossypol is expected to be useful for the treatment of diverse cancer cell types.

7. In another study, gossypol was shown to have antiproliferative effects in both MCF-7 human mammary cancer cells and HT1080 fibrosarcoma cells as measured by [$^3$H]thymidine incorporation into DNA. See Ligueros et al., *British J. of Cancer*, 76(1): 21-28 (1997) (Exhibit C). The study determined that, in the MCF-7 cells, the anti-mitogenic effect of gossypol was cell cycle related. These cells showed a dose-dependent percentage increase of cells in the $G_1$ and S phase, lending support for the conclusion that gossypol reduces the mitotic index of MCF-7 cells. Further, this study showed that in MCF-7 cells, pRb, an important cell cycle protein governing transition from $G_1$ to S phase was decreased by exposure to gossypol in a dose-dependent manner. Cyclin D1/Cdk4 regulates Rb phosphorylation state. Cyclin D1/Cdk4 also was found to be decreased in MCF-7 cells exposed to gossypol, in a dose-dependent manner. Thus, gossypol exhibited an antiproliferative effect in MCF-7 breast cancer cells and HT1080 fibrosarcoma cells. Gossypol further had an arresting effect on the cell cycle of MCF-7 breast cancer cells. Due to the effect of gossypol on cell cycle regulation, There is a reasonable expectation that gossypol would be useful to treat diverse cancer cell types.

7. Adrenal cancer is a rare, fatal malignancy for which medical therapy is largely unsuccessful. However, a study examining the efficacy and toxicity of oral gossypol as a treatment for metastatic adrenal cancer in humans showed that gossypol was useful for the treatment of metastatic adrenal cancer. See Flack et al., *JCE&M*, 74(4): 1019-1024 (1993) (Exhibit D). Twenty-one patients received oral gossypol at doses of 30-70 mg/day. Three of the 18 patients, who had previously failed other chemotherapeutic regimens, had partial tumor responses to gossypol. In fact, in these three patients, one experienced a 90% reduction in the size multiple lung metastases and 80-90% reduction in the volume of

multiple hepatic lesions. The second patient experienced an 80% reduction in tumor volume and the third patient experienced a 50% reduction in tumor volume. This study showed that gossypol was effective as a second line salvage agent in patients with metastatic adrenal cancer who are refractory to other chemotherapeutic agents.

8. Gliomas are infiltrating tumors of the central nervous system that are rarely cured by surgery and radiation therapy. Chemotherapy for gliomas is largely unsuccessful. The efficacy of gossypol was studied in the context of recurrent adult malignant gliomas. See Bushunow et al., *J. of Neuro-Oncology*, 43: 79-86 (1999) (Exhibit E). Gliomas typically have a distinct lactate dehydrogenase (LDH) profile compared to normal brain tissue. Gliomas contain high levels of cationic forms of LDH (i.e., $LDH_4$ and $LDH_5$). As gossypol inhibits the activity of many enzymes, including LDHX and other forms of LDH, it thereby inhibits glycolysis. The activity of oral gossypol acetic acid (10 mg/bid) was studied in 27 patients with pathologically confirmed glial tumors that had recurred after radiation therapy, with some recurrent after radiation and chemotherapy. Although four of the patients withdrew or had to be withdrawn from the study prior to assessment, two patients exhibited partial response, and four patients had stable disease states for 8 weeks or more. One patient exhibited a partial response lasting 78 weeks. The partial response of two heavily pre-treated patients demonstrates that gossypol is an effective treatment of advanced glioma.

9. As gossypol has been shown to exhibit effect via various routes of action, a reasonable expectation exists that gossypol, and in particular, (-)-gossypol, will be effective to treat diverse cancer cell types.

10. All statements made herein of my own knowledge are true. All statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 10/18/06

Marcus Reidenberg, M.D.

# CURRICULUM VITA

Marcus Milton Reidenberg

Born: Philadelphia, Pennsylvania
January 3, 1934

Married: 1957

## CURRENT POSITIONS

| | |
|---|---|
| Cornell University Medical College<br>New York, NY | Professor of Pharmacology<br>Professor Public Health<br>Professor of Medicine and Head,<br>Division of Clinical Pharmacology<br>Assistant Dean, Departmental<br>Associates Program |
| The New York Hospital<br>New York, NY | Attending Physician |
| Rockefeller University<br>New York, NY | Visiting Physician |

## PREVIOUS POSITIONS

| | |
|---|---|
| Cornell University Medical College<br>New York, New York | 1981-1982, Acting Associate Dean<br><br>1976-1980, Professor of Pharmacology/Associate Professor of Medicine, and Head, Division of Clinical Pharmacology |
| The New York Hospital<br>New York, New York | 1975-1980, Associate Attending Physician |
| Rockefeller University<br>New York, New York | 1975-1977, Visiting Associate Physician |
| Cornell University Medical College<br>New York, New York | 1975-1976, Associate Professor of Pharmacology/Associate Professor of Medicine |

| | |
|---|---|
| Temple University<br>Philadelphia, Pennsylvania | 1972-1974, Associate Professor of Pharmacology/Associate Professor of Medicine and Head, Section of Clinical Pharmacology |
| Temple University<br>Philadelphia, Pennsylvania | 1971-1972, Associate Professor of Pharmacology/Assistant Professor of Medicine and Head, Section of Clinical Pharmacology |
| Department of Biochemistry<br>St. Mary's Hospital Medical School<br>London, England | 1969-1970, Visiting Scientist with Professor R.T. Williams |
| Department of Clinical Pharmacology<br>Karolinska Institutet<br>Stockholm, Sweden | 1970, Visiting Scientist with Professor Folke Sjoqvist |
| Temple University<br>Philadelphia, Pennsylvania | 1967-1971, Assistant Professor of of Pharmacology and Internal Medicine |
| | 1965-1967, Assistant Professor of Pharmacology, Instructor of Internal Medicine |
| | 1965, Instructor of Pharmacology, Instructor of Internal Medicine |
| | 1962-1965, Instructor of Pharmacology, Resident in Internal Medicine |
| U.S. Navy | 1960-1962, Senior Medical Officer U.S. Naval Station, Trinidad |
| Temple University<br>Philadelphia, Pennsylvania | 1959-1960, U.S.P.H.S. Postdoctoral Fellowship in Pharmacology with Dr. R. W. Sevy |
| Community General Hospital<br>Reading Pennsylvania | 1958-1959, Internship |

| | |
|---|---|
| Temple University<br>Philadelphia, Pennsylvania | 1955-1958, Summer Student Research Fellow in Physiology and Pharmacology with Drs. E. A. Ohler and R. W. Sevy<br><br>1954-1958, Medical School (M.D., 1958) |
| Cornell University<br>Ithaca, New York | 1951-1954, Pre-medical (no degree) |

## SOCIETY MEMBERSHIP

| | |
|---|---|
| Association of American Physicians | Sigma Xi |
| American Society for Clinical Investigation | The Harvey Society |
| American College of Physicians (Fellow) | The New York Academy of Sciences |
| Society for Experimental Biology and Medicine | The American Society of Nephrology |
| American Society for Pharmacology and Experimental Therapeutics | American Association for the Advancement of Science |
| American Society of Tropical Medicine and Hygiene | Council on Basic Science, American Heart Association |
| American Society for Clinical Pharmacology and Therapeutics | Royal Society of Tropical Medicine and Hygiene |
| American Federation for Clinical Research | Royal Society of Medicine (Fellow) |
| International Association for the Study of Pain | American Geriatrics Society |

## LICENSES AND CERTIFICATIONS AND AWARDS

License to Practice Medicine in Pennsylvania and New York.

Certified by American Board of Internal Medicine, December 7, 1967.

Fred Conrad Koch Travel Fellow of The Endocrine Society Award, 1968.

Research Career Development Award, National Institute of General Medical Sciences, National Institutes of Health, 1970-1974.

Temple University S.A.M.A. Teacher Recognition ("Golden Apple") Award, 1973.

Rawls-Palmer Award of the American Society for Clinical Pharmacology and Therapeutics, 1981.

Experimental Therapeutics Award of the American Society for Pharmacology and Experimental Therapeutics, 1983.

William Creasy Visiting Professor of Clinical Pharmacology at the University of Oregon Medical School, 1981; University of Cincinnati Medical School, 1984.

Julius W. Sturmer Memorial Lecture Award of the Philadelphia College of Pharmacy and Science, 1982.

Cornell University Medical College Second Year Teaching Award, 1986, 1992.

Temple University Medical School Alumni Achievement Award, 1988.

Cornell University Medical College Alumni Association Honorary Fellowship, 1993.

The Departmental Associates of The New York Hospital-Cornell Medical Center established a program to provide minority college students with summer research opportunities and named it the Marcus M. Reidenberg Gateways to Science Program, 1993

Pfizer Visiting Professor of Clinical Pharmacology, Meharry Medical College, 1995

Fellow, American Association for the Advancement of Science, 1995

Cornell University Medical College Senior list teaching award, 1995, 1999, 2001

Henry B. Elliott Award for Distinguished Service of the American Society for Clinical Pharmacology and Therapeutics, 1999.

Harry Gold Award for Research and Teaching Excellence in Clinical Pharmacology of the American Society for Pharmacology and Experimental Therapeutics, 1999.

Elliot Hochstein Teaching Award of the Joan and Sanford Weill Medical College of Cornell University, 1999.

Nathaniel T. Kwit Memorial Distinguished Service Award, American College of Clinical Pharmacology, 1999.

Weill Medical College of Cornell University Award for Teaching Excellence, 2000.

Temple University Medical School 2001 Alumnus of the Year Award.

## EXTRACURRICULAR ACTIVITIES

World Health Organization
- Expert Advisory Panel on Drug Evaluation, Member 1989-1993, 1993-1997, 1997-2001, 2001-2005, 2005-2009
- Expert Committee on the Use of Essential Drugs, Rapporteur, 1989
- Temporary Advisor, WHO International Drug Monitoring Program, 1990
- Expert Committee on the Use of Essential Drugs, Rapporteur, 1991
- Expert Committee on the Use of Essential Drugs, Rapporteur, 1993
- Temporary Advisor, Special Program of Research, Development, and Research Training in Human Reproduction, 1992
- Advisor about Essential Drugs Program, Ministry of Health, People's Republic of China, 1993
- Expert Committee on the Use of Essential Drugs, 1995, 2002
- Expert Committee on the Use of Essential Drugs, Rapporteur, 1999
- Plenary Lecturer, Silver Anniversary meeting of national Centers participating in WHO Programme for International Drug Monitoring, 2002
- Expert Committee on the Selection and Use of Essential Medicines, Vice-Chairman, 2003, 2005

American College of Physicians
- Chairman, Ad Hoc Committee on Clinical Pharmacology, 1971
- Chairman, Committee on Clinical Pharmacology, 1972-1977
- Member, MKSAP VI Committee, 1980-1982
- Director, C.M.E. Course, Individualization of Drug Therapy, 1973
- Director, C.M.E. Course, The Clinical Pharmacology of Symptom Control, 1982

American Society for Pharmacology and Experimental Therapeutics
- Member of Executive Committee, Division of Clinical Pharmacology, 1971-1978, 1980-

Acting Chairman, Executive Committee, Division of Clinical Pharmacology, 1972
Membership Committee, 1979-1982; Chairman, 1981-1982
Educational Affairs Committee, Chairman of Subcommittee on Continuing Medical Education, 1980-1981
Program Chairman for Second World Congress on Clinical Pharmacology, Washington, D.C., July-August 1983
Nominating Committee, 1984
Member, Board of Publications Trustees, 1985- 2001
Member, Harry Gold Award Committee 2000-2001, Chair 2001-2004

International Union of Pharmacology
Member, Working Group on Drug Metabolism, 1982-1984
Vice Chairman, Section on Clinical Pharmacology, 1984-1987
Chairman, Section of Clinical Pharmacology, 1987-1989
Immediate Past Chairman, Section of Clinical Pharmacology, 1989-1992
Member, Working Group to Review IUPHAR Statutes, 1988
Auditor, 1990-1993
Member, International Advisory Committee, World Conference on Clinical Pharmacology of 1992, 1996
Member, Membership Committee, 1990-1993, 1994-1997, 1997-2001

American Society for Clinical Pharmacology and Therapeutics
Board of Directors, 1975-1977, 1979-1982, 1986-2001
Vice President, 1982-1983
President Elect, 1983-1984
President, 1984-1985
Multiple Committee Memberships, 1980-2001

Consultant, South-to-South Cooperation in Reproductive Health, 1991-1999

Chairman, Session on Clinical Pharmacokinetics, Gordon Research Conference on Drug Metabolism, 1971 and 1976

National Academy of Sciences
Consultant to Drug Research Board, 1968-1970
Speaker. I.O.M. Workshop on Pharmacokinetics and Drug Interactions in the Elderly, 1997

Food and Drug Administration
Consultant, 1971
Member, Project Advisory Group, Experiment in Early Postmarketing Surveillance of Drugs, 1977-1982
Invited Expert, Fertility and Maternal Health Drugs Advisory Committee, 1990
Member, Over-The-Counter (OTC) Drugs Advisory Committee, 1992-1995.

Anesthetic and Life support Drugs Advisory Committee, Member 2001
Invited Participant, CPDD, FDA, DEA, NIH workshop on Abuse Liability Assessment of CNS Drugs, 2002.

Consultant, Walter Reed Army Medical Center, 1974-1977

National Institutes of Health
Special Study Section, 1980 and 1986
Task Force on Geriatric Medicine, 1980
Pharmacological Sciences Review Committee (Study Section), 1980-1985
Workshop Chairman, Program Initiatives in Gerontological Pharmacology 1981
Consensus Conference on Pain Management Panel Member, 1986
Epidemiology and Disease Control Special Study Section, 1990
Data Safety Monitoring Review Committee for MIRA trial, 1991-1993

Joint Commission on Prescription Drug Use, Member, 1976; Vice Chairman, 1977-1980

Consultant, Hoffmann-La Roche, Inc., 1975-

Advisory Board, Orphan Medical, 1994-2001

Consultant, Genta, Inc. 1994-1996

Consultant, Bristol Myers Squibb, 1995-2001

Consultant, Hoechst Marion Roussel, 1996-1998

Association of American Medical Colleges Ad Hoc Committee on AAMC/FDA Interactions, 1978-1982

Merck Company Foundation
International Clinical Pharmacology Fellowship Awards Selection Committee 1992-1995, Chairman 1994-1996

American Federation for Aging Research
Research Committee 1986-1992
Fellowship Selection Committee 1993, 1994, 1995, 1997, 1998, 1999, 2000, 2001

Award Committee for Nellie Westerman Prize for Research in Ethics of the American Federation for Clinical Research, 1975-1978

USP - Delegate from Cornell University Medical College, 1975-1980
Advisory Panel on Geriatrics, Committee on Revision, 1980-1990

Consultant, Office of Technology Assessment, Congress of the United States, 1981

Consultant, New York State Department of Health, 1988-1992

Vice Chairman, Greater Philadelphia Committee for Medical-Pharmaceutical Sciences, 1968-1974

Representative of Pennsylvania Medical Society to Task Force on Drug Handling of the Commonwealth of Pennsylvania, 1968

UNICEF
Emergency Operations in former Yugoslavia, Advisor for Manual for Essential Drugs, 1994

USP DI-AMA DE Advisory Council, 1995-1997

Médecins Sans Frontieres (Doctors Without Borders)
Participant in workshop, The Crisis of Neglected Diseases: Developing Treatments and Ensuring Access, 2002

Organizer, Esteve Foundation Workshop on Peer Review of Scientific Publications, 2002

## SCIENTIFIC PUBLICATIONS

Associate Editor, Clinical Pharmacology and Therapeutics, 1980-1984
Editor, Clinical Pharmacology and Therapeutics, 1985-2001
Editor, Emeritus, Clinical Pharmacology and Therapeutics, 2002-

## EDITORIAL BOARDS

Primary Care, 1974-1979
Journal of Dialysis, 1976-1985
Clinical Pharmacokinetics, 1976-1997
Archives Internationales de Pharmacodynamie et de Therapie, 1976-1995
Pharmacology, 1978-1985
Clinical Pharmacology and Therapeutics, 1978-1980
Rational Drug Therapy, 1978-1985
Renal Physiology, 1978-1985
Therapeutic Drug Monitoring, 1978-2001
Trends in Pharmacological Sciences, 1978-2000
Clinical Nephrology, 1978-1995

## BOOKS

Reidenberg, M.M.: Renal Function and Drug Action. Philadelphia, PA, W.B. Saunders Co., 1971.

Reidenberg, M.M., editor: Individualization of Drug Therapy. Medical Clinics of North America, Philadelphia, PA, W.B. Saunders Co., September, 1974.

Rubin, A.L., Stenzel, K.H., and Reidenberg, M.M., editors: Symposium on Drug Action and Metabolism in Renal Failure. Amer. J. Med., 62:459-563, 1977.

Reidenberg, M.M., editor: The Clinical Pharmacology of Symptom Control. Medical Clinics of North America, Philadelphia, PA, W.B. Saunders Co., September, 1982.

Lemberger, L. and Reidenberg, M.M., editors: Proceedings of Second World Conference on Clinical Pharmacology and Therapeutics. American Society for Pharmacology and Therapeutics, Bethesda, MD, 1984.

Reidenberg, M.M. and Erill, S., editors: Drug-Protein Binding. Praeger, New York, NY, 1986.

Reidenberg, M.M., editor: The Clinical Pharmacology of Biotechnology Products. Excerpta Medica, New York, NY, 1991.

Sirtori C, Kuhlmann J, Tillement J-P, Vrhovac B, Reidenberg MM., eds. Clinical Pharmacology. McGraw-Hill, London, 2001.

## Publications

### Original Research in Refereed Journals

1. Hartman, J.D. and Reidenberg, M.M.: Comparison of the glycolytic activity of blood and exudate leucocytes. J. Appl. Physiol., 12:477-481, 1958.

2. Reidenberg, M.M., Ohler, E.A., and Sevy, R.W.: Cardiovascular responses to norepinephrine in acute adrenal insufficiency. Proc. Soc. Exper. Biol. and Med., 97:889-892, 1958.

3. Soloff, L.A., Reidenberg, M.M., Winters, W.L. Jr., and Bello, C.T.: Clinical experiences with bretylium tosylate. Ann. N.Y. Acad. Sci., 88(4):1003-1010, 1960.

4. Reidenberg, M.M. and Sevy, R.W.: Effect of adrenocortical steroids on bone electrolyte metabolism. Proc. Soc. Exper. Biol. and Med., 107:132-134, 1961.

5. Reidenberg, M.M., Ohler, E.A., Sevy, R.W., and Harakal, C.: Hemodynamic changes in adrenalectomized dogs. Endocr., 72:918-923, 1963.

6. Reidenberg, M.M. and Barry, W.E.: Low molecular weight dextran. Lancet, 1:988-989, 1964.

7. Adler, M.W., Reidenberg, M.M., Harakal, C., Rusq, B.F., and Papacostas, C.A.: Cardiovascular effects of hexafluorodiethyl ether. Int. J. Neuropsychiatry, 1:511-512, 1965.

8. Harakal, C., Reidenberg, M.M., Sevy, R.W., and Ohler, E.A.: Hemodynamic effects of adrenal medullectomy in the dog. Am. J. Physiol., 210:5-6, 1966.

9. Reidenberg, M.M., Haag, B.L., Channick, B.J., Shuman, C.R., and Wilson, G.G.: The response of bone to metabolic acidosis in man. Metabolism, 15:236-241, 1966.

10. Molthan, L., Reidenberg, M.M., and Eichman, M.F.: Positive direct Coombs' tests due to cephalothin. New Engl. J. Med., 277:123-125, 1967.

11. Barrera, F., Reidenberg, M.M., and Winters, W.L.: Pulmonary function in obese patients. Am. J. Med. Sci., 254:785-796, 1967.

12. Haag, B.L., Reidenberg, M.M., Shuman, C.R., and Channick, B.J.: Aldosterone, 17-ketosteroid, and fluid and electrolyte responses to starvation and selective refeeding. Am. J. Med. Sci., 254:652-658, 1967.

13. Reidenberg, M.M.: Registry of adverse drug reactions. Report of the drug reaction registry subcommittee of The Greater Philadelphia Committee for Medical-Pharmaceutical Sciences. J.A.M.A., 203:31-43, 1968.

14. Reidenberg, M.M., Sevy, R.W., and Cucinotta, A.J.: Hypercalciuria during acidosis in hypoparathyroidism. Proc. Soc. Exper. Biol. and Med., 127:1-3, 1968.

15. Harakal, C., Sevy, R.W., Reidenberg, M.M., and Faust, R.E.: Effect of adrenal medullectomy and total adrenalectomy on the hemodynamic responses to tyramine. J. Pharmacol. Exper. Ther., 160:292-299, 1968.

16. Reidenberg, M.M. and Lowenthal, D.T.: Adverse nondrug reactions. New Engl. J. Med., 279:678-679, 1968.

17. Reidenberg, M.M., Kostenbauder, H., and Adams, W.P.: The rate of drug metabolism in obese volunteers before and during starvation and in azotemic patients. Metabolism, 18:209-213, 1969.

18. Barrera, E.F., Reidenberg, M.M., Winters, W.L., and Hungspreugs, S.: Ventilation perfusion relationships in the obese patient. J. Appl. Physiol., 26:420-426, 1969.

19. Magargal, L.E., Magargal, H., and Reidenberg, M.M.: Effect of steroid hormones on the parathyroid hormone dose-response curve. J. Pharmacol. and Exper. Ther., 169(1):138-141, 1969.

20. Coleman, E.H. and Reidenberg, M.M.: Effect of thyroparathyroidectomy on skeletal sodium in the rat. Endocrinology, 85:175-176, 1969.

21. Glauser, S.C., Glauser, E.M., Reidenberg, M.M., Rusy, B.F., and Tallarida, R.J.: Metabolic changes associated with the cessation of cigarette smoking. Arch. Environ. Health, 20:377-381, March, 1970.

22. Reidenberg, M.M., Odar-Cederlof, I., Von Bahr, C., Borga, O., Sjoqvist, F.: Protein binding of diphenylhydantoin and desmethylimipramine in plasma from patients with poor renal function. New Engl. J. Med., 285:264-267, 1971.

23. Lowenthal, D.T. and Reidenberg, M.M.: The heart rate response to atropine in uremic patients, obese subjects before and during fasting, and patients with other chronic illnesses. Proc. Soc. Exper. Biol. and Med., 139(2):390-393, 1972.

24. Reidenberg, M.M., James, M., and Dring, L.G.: The rate of procaine hydrolysis in serum from normal subjects and diseased patients. Clin. Pharmacol. and Ther., 13:279-284, 1972.

25. James, M., Smith, R.L., Williams, R.T., and Reidenberg, M.M.: The conjugation of phenylacetic acid in man, subhuman primates, and some non-primate species. Proceedings of The Royal Society B, 182:25-35, 1972.

26. Reidenberg, M.M.: The procaine esterase activity of serum from different mammalian species. Proc. Soc. Exper. Biol. and Med., 140:1059-1061, 1972.

27. Drayer, D.E. and Reidenberg, M.M.: Metabolism of tetralin and toxicity of Cuprex(R) in man. Drug Metabolism and Disposition, 1:577-579, 1973.

28. Lowenthal, D.T., Chardo, F., and Reidenberg, M.M.: Removal of mercury by peritoneal dialysis. Arch. Int. Med., 134:139-141, 1974.

29. Reidenberg, M.M., Drayer, D.E., DeMarco, A.L., and Bello, C.T.: Hydralazine elimination in man. Clin. Pharmacol. and Ther., 14:970-977, 1973.

30. Reidenberg, M.M., Shear, L., and Cohen, R.V.: Elimination of isoniazid in patients with impaired renal function. Am. Review of Respir. Dis., 108:1426-1428, 1973.

31. Reidenberg, M.M. and Martin, J.H.: The acetylator phenotype of patients with systemic lupus erythematosus. Drug Metabolism and Disposition, 2:71-73, 1974.

32. Kessler, K.M., Lowenthal, D.T., Warner, H., Gibson, T., Briggs, W., and Reidenberg, M.M.: Quinidine elimination in patients with congestive heart failure or poor renal function. New Engl. J. Med., 290:706-709, 1974.

33. Drayer, D.E., Reidenberg, M.M., and Sevy, R.W.: N-acetylprocainamide: An active metabolite of procainamide. Proc. Soc. Exper. Biol. and Med., 146:358-363, 1974.

34. Drayer, D.E., Strong, J.M., Jones, B., Sandler, A., and Reidenberg, M.: In vitro acetylation of drugs by human blood cells. Drug Metabolism and Distribution, 2:499-505, 1974.

35. Affrime, M. and Reidenberg, M.M.: The protein binding of some drugs in plasma from patients with alcoholic liver disease. Eur. J. Clin. Pharmacol., 8:267-269, 1975.

36. Reidenberg, M.M., Drayer, D.E., Levy, M., and Warner, H.: The polymorphic acetylation of procainamide by man. Clin. Pharmacol. Ther., 17:722-730, 1975.

37. Reidenberg, M.M. and Vesell, E.S.: Unaltered metabolism of antipyrine and tolbutamide during fasting in man. Clin. Pharmacol. Ther., 17:650-656, 1975.

38. Reidenberg, M.M. and Caccese, R.W.: Lymphocyte transformation tests and suspected drug allergy. J. Lab. Clin. Med., 86:997-1002, 1975.

39. Cerletti, C., Keinath, S.H., Reidenberg, M.M., and Adler, M.: Relationship between method of morphine administration, plasma levels, and the withdrawal syndrome in rats. Pharmacol., Biochem., and Behavior, 4:323-327, 1976.

40. Bagwell, E.E., Walle, T., Drayer, D.E., Reidenberg, M.M., Pruett, J.K.: Correlation of the electrophysiological and antiarrhythmic properties of the N-acetyl metabolite of procainamide with plasma and tissue drug concentrations in the dog. J. Pharmacol. Exper. Ther., 197:38-48, 1976.

41. White, R.P., Sealey, J., Reidenberg, M.M., Stenzel, K.H., Sullivan, J.F., David, D.S., Laragh, J.H., and Rubin, A.L.: Mechanisms of blood pressure control in anephrics: plasma renin and dopamine B hydroxylase activity. Trans. Amer. Soc. Artif. Int. Organs, 22:420-423, 1976.

42. Chami, J., Reidenberg, M., Wellner, D., David, D.S., Rubin, A., and Stenzel, K.H.: Essential amino acid metabolism in maintenance dialysis patients. Trans. Amer.Soc. Artif. Int. Organs, 22:168-173, 1976.

43. Reidenberg, M.M., Lowenthal, D.T., Briggs, W., and Gasparo, M.: Pentobarbital elimination in patients with poor renal function. Clin. Pharmacol. Ther., 20:67-71, 1976.

44. Drayer, D.E., Cordova, M., Slaven, B.H., Bagwell, E.E., and Reidenberg, M.M.: The antiarrhythmic activity of p-hydroxy-N-(2-diethylaminoethyl) benzamide (the p-hydroxy isostere of procainamide) in dogs and mice. J. Med. Chem., 20:270-274, 1977.

45. Tapia, L., Cheigh, J.S., David, D.S., Sullivan, J.F., Saal, S., Reidenberg, M.M., Stenzel, K.H., and Rubin, A.L.: Treatment of pruritus in dialysis patients with parenteral lidocaine. New Engl. J. Med., 296:261-262, 1977.

46. Szeto, H.H., Inturrisi, C.E., Houde, R., Saal, S., Cheigh, J., Reidenberg, M.M.: Accumulation of normeperidine, an active metabolite of meperidine, in patients with renal failure or cancer. Ann. Int. Med., 86:738-741, 1977.

47. Drayer, D.E., Lowenthal, D.T., Woosley, R.L., Nies, A.S., Schwartz, A., and Reidenberg, M.M.: Cumulation of N-acetylprocainamide, an active metabolite of procainamide, in patients with impaired renal function. Clin. Pharmacol. Ther., 22:63-69, 1977.

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# Modulating the Bcl-2 Family of Apoptosis Suppressors for Potential Therapeutic Benefit in Cancer

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**Members of the BCL-2 family of proteins regulate and execute many cell intrinsic apoptosis pathways, including those arising from dysregulated expression of cellular oncogenes. Since pro-survival members of the family are often strongly elevated in diverse cancers, with the potential to confer resistance to both endogenous cell death stimuli and many cancer treatments, there has been intense interest to develop strategies to therapeutically modulate their activity. Although encouraging genetic and pharmacological preclinical proof of concept has been obtained, the challenge for clinical development will be to devise strategies that address the fact that multiple pro-survival members are typically up-regulated in a given cancer and the family operates primarily through protein-protein interactions. Moreover, since several current therapies themselves are known to stimulate the levels of one or more family members, there will be additional challenges (and opportunities) in exploiting this target in the clinic. In this review, we describe the rationale for targeting the BCL-2 family of apoptosis suppressors in cancer and the progress that has been made in modulating the family by small molecule antagonists.**

Apoptosis is believed to have evolved in metazoans to regulate tissue homeostasis and to eliminate individual cells that have become superfluous, have become dysfunctional due to infections, or sustained chromosomal alterations that could subvert normal growth control. Apoptosis therefore provides a defense against numerous assaults that could otherwise inflict damage or kill the organism. During oncogenesis the cell must bypass these inherent apoptosis mechanisms if the cancer cell is to survive because apoptosis is otherwise triggered by both the aberrant growth pattern of these cells and the application of many cancer therapies. Cancer cells can evade apoptosis in either of two ways: by inactivation of genes or gene products that promote apoptosis (e.g., the p53 tumor suppressor gene) or by activation of inhibitors of cell death pathways (e.g., the BCL-2 family of apoptosis suppressors). Therapies that target these regulators and re-instate the normal apoptotic mechanisms in cancer cells hold significant promise.[1-3]

## Oncogenes as Inducers of Apoptosis Pathways

Unrestricted mitogenic stimuli arising from dysregulated oncoprotein signaling is an early step in conferring a predisposition to malignant transformation, a condition that is normally held in check by interlocking tumor suppressor mechanisms, usually resulting in apoptosis.[4,5] The concept that transforming oncogenes can stimulate apoptosis mechanisms is now well established in many contexts, and includes cell membrane signaling by Ras[6] or transcriptional changes effected by Myc.[7,8] Animal models have been engineered for pancreatic beta cell oncogenesis in which a combination of c-Myc expression and upregulation of a suppressor of apoptosis, BCL-$X_L$, is both necessary and sufficient to permit c-Myc-induced initiation and progression of cells into angiogenic, invasive tumors.[9] Conditional activation of c-Myc in adult, mature beta cells in this transgenic mouse model in the absence of an apoptosis suppressor, on the other hand, induced uniform beta cell proliferation but was accompanied by massive apoptosis, which rapidly degraded the beta cell mass. Conversely, in a mouse lymphoblastic leukemia model driven by constitutive c-Myc[10] and conditional BCL-2 expression, subsequent elimination of BCL-2 yielded rapid loss of leukemic cells and significantly prolonged survival. After turning off BCL-2, the oncogenic potential of c-Myc was overcome by apoptosis, formally validating inhibitors of BCL-2 as a rational strategy for therapeutic development. Based on these and related findings it has been proposed that a combination of dysregulated cell proliferation and reduction in apoptosis is key for the development of cancer, with the secondary traits of diverse neoplasms resulting as outcomes of this platform.[9,11]

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Acknowledgments: We thank Pierre Beauparlant for reviewing the manuscript and providing input.

## BCL-2 Family of Death Regulators

The BCL-2 family is central to both the regulation and execution of most intrinsic apoptotic pathways.[12] The family is comprised of 3 groups, classified according to their content of BCL-2 homology (BH) domains (for a recent detailed review, see [12]). Anti-apoptotic members (e.g., BCL-2, BCL-$X_L$, BCL-w, MCL-1, and A1) contain four BH domains defined by their similarity among the members of the family; the multi-BH domain pro-apoptotic members BAX and BAK contain BH domains 1-3; and a diverse group of loosely related pro-apoptotic proteins (e.g., BID, BAD, BIM, BIK, PUMA, NOXA, etc.) contain only BH domain 3 (BH3). All anti-apoptotic members as well as BAX and BAK contain a hydrophobic transmembrane (TM) domain located at their extreme C-terminus, whereas among BH3-only members BIK, BIM, and PUMA contain a C-terminal TM. Anti-apoptotic members have the potential to hetero-dimerize with pro-apoptotic members through binding of the exposed BH3 helix on the surface of pro-apoptotic members into a deep groove on the surface of anti-apoptotic members, formed by helices 1 and 2.[13] BAX and BAK, as well as certain BH3 only proteins, undergo a conformational change in response to upstream death signaling pathways, resulting in exposure or availability of the BH3 domain. Heterodimerization with anti-apoptotic BCL-2 members, therefore, typically occurs with activated pro-apoptotic conformers.[14-16] Of note, a number of BH3 only proteins, including PUMA, NOXA and BIK exist as constitutively active conformers and therefore their contribution to death signaling necessarily involves new protein synthesis. In addition, certain BH3-only proteins can interact, at least transiently with BAX or BAK.[14-16] The outcome of death signals that are regulated by the BCL-2 family, therefore, depends upon a complex three-way ratio of the multi-domain anti-apoptotic, multi-domain pro-apoptotic, and BH3-only members (**Figure 1**).

Studies employing double gene deletions of murine Bax and Bak have shown that these two proteins function as essential effector molecules in many death pathways[17] and that the anti-apoptotic BCL-2 and pro-apoptotic BH3-only members operate both upstream and through these effector molecules.[18] The BH3-only members function as proximal sensors of apoptotic stimuli and in their active conformers can bind and inhibit BCL-2 members (e.g., BAD and NOXA) or they can both inhibit BCL-2 members as well as activate BAX and BAK by a "hit-and-run" mechanism (e.g., tBID and BIM). The former act as sensitizers of stimuli that activate BAX and BAK, whereas the latter are both sensitizers and activators.[15]

As depicted in **Figure 1**, the ratio between pro-survival BCL members and pro-death members dictates the outcome of many death-initiating signalling pathways. To achieve this, the BCL-2 family functions at two sites within the cell: mitochondria, where the BCL members regulate the release of factors from the organelle that activate caspases and remodel chromatin; and endoplasmic reticulum (ER), where the BCL members regulate ER $Ca^{2+}$ homeostasis and release.[19] The BCL-regulated ER $Ca^{2+}$ pathway is linked to the mitochondria, causing morphological and structural transitions that allow mitochondria to respond to pro-apoptotic stimuli.[20] Of note, pro-survival BCL members are differentially enriched at mitochondria and ER.[21] **Figure 2** (see Color Figures, page 552) illustrates the pathway at mitochondria, in which an oncogenic stimulus results in activation of one or more BH3-only members, which can target and antagonize pro-survival members. Productive antagonism of pro-survival members either alone or coupled with stimuli to directly activate BAX and BAK, results in the oligomerization of BAX or BAK. This allows the formation of a predicted conduit for release of pro-apoptotic factors such as cytochrome c, a co-factor that results in activation of the apoptosome, which in turn activates effector caspases –3 and –7.[12]

## Not All BH3 Domains Are Created Equal

Individual BH3-only BCL-2 members appear to have evolved both to link specific upstream signals to downstream activation of the mitochondrial apoptosis pathway and to selectively target preferred BCL-2 binding partners. For example a recent study of the affinity of 8 BH3 peptides for the soluble forms of 5 pro-survival BCL-2 proteins, employing a Biacore Biosensor, revealed a 10,000-fold range in binding affinity.[22] BIM and PUMA, for example, exhibited similar affinities for all pro-survival members whereas NOXA bound only to MCL-1 and A1. BH3-only BIK, which can be induced by oncogenic stress, preferentially targets the ER where it binds pro-survival members and initiates $Ca^{2+}$-mediated remodelling of mitochondrial cristae, mobilizing mitochondrial stores of cytochrome c as a prerequisite for its release to the cytosol.[20] Interestingly, BIK and NOXA cooperate to release cytochrome c.

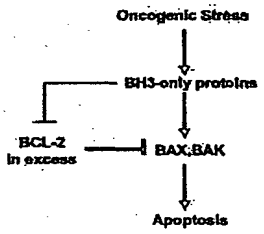


**Figure 1. Model for regulation of oncogene-driven apoptosis by BCL-2 family proteins.** Oncogenic stress pathways lead to activation of several BH3-only proteins. They all antagonize pro-survival BCL-2 members but in addition certain of these BH3-only members also activate BAX and BAK. When in excess, pro-survival members prevent apoptosis by antagonizing the activated conformers of BAX and BAK. Therapeutics that shift the balance in favor of excess antagonists of pro-survival members should permit oncogenic stress pathways to productively engage the apoptosis mechanism. Details are given in the text.

Likewise, BAD and NOXA, which have non-overlapping binding preferences for pro-survival members, cooperate to induce cell killing.[22] From a therapeutic perspective, it might be expected that mimetics of the BH3 domains of BIM and PUMA exhibit pan-BCL inhibition, whereas more selective antagonists might be generated from other BH3 mimetics, such as those derived from BAD or NOXA.

## Pro-survival BCL-2 Proteins: Therapeutic Targets

Since pro-apoptotic BCL family proteins dock into the BH1-BH2 groove of pro-survival members via their BH3 domain, it has been proposed that BH3 mimetics that antagonize the pro-survival members could be used to alter the ratio between pro-survival and pro-apoptotic members, allowing apoptosis to proceed in cancer cells. Strong support for such a strategy has come from the findings that BH3 peptides themselves can inhibit BCL members, induce apoptosis in cancer cell lines, and in one case where the pharmacological properties of the peptide were improved, induce apoptosis in a mouse xenograft tumor model.[23] In this latter case, a chemical strategy termed hydrocarbon stapling was used to stabilize the alpha-helical BH3 peptide derived from the BH3-only protein BID. BID is strongly pro-apoptotic and belongs to the class that activates BAX and BAK as well as antagonizes pro-survival BCL members.[23] The stapled peptide proved to be cell permeant and protease-resistant, and interacted with pro-survival members with increased affinity. It was also effective in inhibiting the growth of human leukemia xenografts in SCID mice. Thus, a BH3 peptide, and therefore small molecules that mimic this domain, has the potential to therapeutically modulate BCL-2 family proteins.

## Therapeutic Small Molecule Discovery Strategies

The challenge of this strategy is to discover corresponding small molecule BCL antagonists with drug-like properties. Moreover, because of the complexity of BCL proteins in cancer cell biology, including upregulation of multiple family members in a single cell and the contribution of different family members to mitochondria- and ER-regulated pathways, a small molecule antagonist of multiple pro-survival members is likely required, i.e., a pan-BCL-2 inhibitor, at least for initial proof of concept studies in the clinic. Although a number of such antagonists are currently at various stages of development, we focus below on two examples representing distinct discovery strategies: rational design and functional screening.

### *ABT-737*

One approach is based on rational design and high throughput SAR by NMR.[23] Utilizing the high resolution structure of the BH3 docking groove on the surface of the pro-survival BCL member, BCL-$X_L$,[13] inhibitors of BCL-$X_L$, BCL-2, and BCL-w were generated by covalently bridging chemical entities that bind to separate regions of the groove. One, ABT-737, exhibits an affinity for these targets in vitro 2- to 3-orders of magnitude more potent than the multiple small molecule antagonists that have previously been reported in the literature ([24] and references cited therein). Of note, however, it exhibits significantly reduced affinity for MCL-1, a BCL member whose structure is intermediate between the "closed" conformation of unliganded BCL-2/BCL-$X_L$ and their more "open" BH3-complexed conformers.[25] A number of amino acids in the binding groove also distinguish MCL-1 from other members. Nevertheless, ABT-737 demonstrated potent single-agent killing of select cell lines from small cell lung carcinoma and lymphoma, and against peripheral blood mononuclear cells (PBMNCs) derived from 7 of 13 patients with chronic lymphocytic leukemia (CLL). From a mechanistic perspective, ABT-737 appears to fall into the class of BH3 "sensitizers," since it fails to directly activate BAX or BAK and release cytochrome c from mitochondria in vitro.[24] Despite the relatively large size of the compound, ABT-737 achieved potent anti-tumor activity in mouse H146 and H1963 small cell lung carcinoma (SCLC) mouse xenograft models when administered i.p. at 75-100 mg/kg daily for 3 weeks.

### *GX15-070*

An alternative discovery approach is based on functional outcomes and seeks small molecules that inhibit BCL protein-protein interactions. Since BCL members have the potential to undergo conformational changes,[14,16,27] these assays accommodate the possibility of dynamic changes in protein structure contributing to these interactions. Thus, a high throughput protein-protein interaction discovery screen was used to interrogate natural compound libraries, which identified a chemotype that falls within the polypyrrole class of molecules[28] as a starting point for optimization. Further development resulted in the compound GX15-070, a non-prodigiosin, which is currently in clinical development.

[$^3$H]-labelled GX15-070 was found to bind to BCL-w, BCL-$X_L$, and MCL-1 with $K_D$ values in the 0.5 μM range. In contrast to ABT-737, therefore, GX15-070 appears to bind pro-survival members as purified entities in vitro with apparent reduced affinity. However it also targets MCL-1. After exposure of sk-MEL5 melanoma cells to GX15-070 for 5 hours and detergent extraction, interaction between MCL-1 and BAK was inhibited relative to vehicle controls, as judged by co-immunoprecipitation, with an apparent $IC_{50}$ of about 1.5 μM.

To formally prove that GX15-070 can antagonize pro-survival BCL members, resulting in activation of BAX or BAK, the BCL pathway was engineered into yeast cells. *S cerivisiae* does not express BCL-related proteins and is not sensitive to GX15-070-mediated cytotoxicity. In contrast, overexpression of BAK in these yeast is cytotoxic, but can be countered by pro-survival members BCL-w, MCL-1, or BCL-$X_L$. However, treatment of the yeast cells with GX15-070 was toxic, an effect dependent on the presence of BAK suggesting that GX15-070 can antagonize the pro-survival

BCL proteins, overcoming their ability to inhibit BAK. Consistent with this mechanism, when transformed baby mouse kidney epithelial cells expressing adenovirus E1A and dominant-negative p53 and derived from either wt mice or mice doubly deleted of *Bax,Bak*, the double knock out cells resisted the activation of caspases by GX15-070. As expected, treatment of cancer cell lines with GX15-070 resulted in oligomerization of mitochondrial BAK, release of cytochrome c, and activation of caspases. Collectively, the results suggest the mechanism illustrated in **Figure 2** (lower panel; see Color Figures, page 552) for the activation of caspases by GX15-070.

Further testing showed that GX15-070 exhibits single agent cytotoxicity against a broad range of cell lines and ex vivo against PBMNCs from patients ($n > 30$) with CLL. Delivery of formulated drug by intravenous bolus injection into the tail veins of Balb/c or CB17 SCID/SCID mice daily for 5 consecutive days was well tolerated, and in animals pre-implanted subcutaneously with cell lines derived from cervical (C33A), colon (SW480), prostate (PC3), or mammary (4T1) carcinomas and allowed to form palpable tumors, administration of GX15-070 on this schedule resulted in inhibition of tumor growth relative to vehicle alone. For example, at 2 mg drug/kg body weight given daily for 5 days, inhibition of growth of these tumors ranged from 60%-85% 14 days after initiating the administration of drug, with no weight loss observed in the animal cohorts. Thus, as predicted from the mechanism of action of BCL proteins and their ability to antagonize oncogenic apoptotic pathways, GX15-070 exhibits antitumor activity as a single agent across diverse cancer cell types.

Phase I evaluation of GX15-070, administered by intravenous infusion on an every 3 week schedule in patients with refractory CLL and weekly in patients with refractory solid tumors, is in progress.

## Pharmacodynamic Markers for BCL-2 Mechanism-Based Cancer Treatments

Evidence of mechanistic and biological activities of BCL mechanism-based therapies can be obtained by measuring these activities directly in cancer cells isolated from the patient as well as by measuring surrogate markers released into the circulation. As indicated, PBMNCs isolated from patients with CLL and incubated with GX15-070 or ABT-737 ex vivo underwent apoptosis. In the case of GX15-070, evidence of disruption of interactions between MCL-1 and BAK was observed following cell extraction in detergent and co-immunoprecipitation. Similar protein-protein interaction assays can be performed on circulating leukemia cells isolated from patients at timed intervals after receiving the drug by intravenous administration. Additionally, end products of apoptosis such as chromatin fragments can be detected following their release into the circulation,[29] thereby serving as a surrogate of tumor cell death. Collectively the results of such biological measurements can be exploited to optimize dose and schedule of drug administration.

## Rational Combination Treatments

Since BCL-2 proteins confer resistance to most cell death stimuli that operate through the mitochondria apoptosis pathway, it is predicted that a number of current cytotoxic cancer treatments might benefit from combination therapy with BCL-2 antagonists. For example, among others, ABT-737 has been reported to enhance the cytotoxicity of paclitaxel in A549 NSCLC cells.[26] Additionally, however, certain current therapies themselves can directly influence the expression of BCL-2 family proteins. The proteasome inhibitor Velcade® (Bortezomib, PS-341; Millennium Pharmaceuticals) is currently approved for the treatment of multiple myeloma and is in development for other indications.[30] By blocking ubiquitin-mediated protein degradation, Bortezomib is predicted to interfere with, among others, survival mechanisms associated with nuclear factor (NF)-κB pathways. It has also been shown to cause elevation of BH3-only NOXA,[31,32] a preferred binding partner for MCL-1.[22] However, at steady state the turnover of MCL-1 is rapid, and this protein also is subject to ubiquitin-mediated degradation via the proteasome.[33] Indeed, proteasome inhibitors can lead to a rapid increase in MCL-1 protein levels in various cell lines within several hours of treatment. If the rise in anti-apoptotic MCL-1 is not offset by pro-apoptotic NOXA (or other BH3 ligand), then a combination of Bortezomib and an effective small molecule antagonist of MCL-1 may prove beneficial.

## Conclusions

The complex interplay between multiple BH3-only proteins and their pro-survival binding partners raises both challenges and opportunities in devising small molecule BH3 therapeutic mimetics for the treatment of cancer. Since more than one pro-survival member is typically overexpressed in a given cancer, early exploration of preclinical and clinical proof of concept is focusing on small molecule antagonists that target multiple pro-survival members. As our understanding of the role of individual pro-survival members in cancer signalling improves, it may prove desirable in certain contexts to design more selective antagonists. The fact that different BH3-only proteins have evolved to accomplish such selectivity suggests that this may indeed be feasible. Coupled with this, however, will be the need to better understand the differential contributions that individual pro-survival members make to cancer-related apoptosis pathways, and to devise the pharmacodynamic and biomarker tools necessary to exploit these opportunities clinically. The clinical development of first generation BCL-2 family antagonists may teach us valuable information about the most effective way to modulate this important family of apoptosis suppressors.

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British Journal of Cancer (1997) 76(1), 21–28


# Gossypol inhibition of mitosis, cyclin D1 and Rb protein in human mammary cancer cells and cyclin-D1 transfected human fibrosarcoma cells

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**Summary** The antiproliferative effects of gossypol on human MCF-7 mammary cancer cells and cyclin D1-transfected HT-1060 human fibrosarcoma cells were investigated by cell cycle analysis and effects on the cell cycle regulatory proteins Rb and cyclin D1. Flow cytometry of MCF-7 cells at 24 h indicated that 10 μM gossypol inhibited DNA synthesis by producing a $G_1$/S block. Western blot analysis using anti-human Rb antibodies and anti-human cyclin D1 antibodies in MCF-7 cells and high- and low-expression cyclin D1-transfected fibrosarcoma cells indicated that, after 6 h exposure, gossypol decreased the expression levels of these proteins in a dose-dependent manner. Gossypol also decreased the ratio of phosphorylated to unphosphorylated Rb protein in human mammary cancer and fibrosarcoma cell lines. Gossypol (10 μM) treated also decreased cyclin D1-associated kinase activity on histone H1 used as a substrate in MCF-7 cells. These results suggest that gossypol might suppress growth by modulating the expression of cell cycle regulatory proteins Rb and cyclin D1 and the phosphorylation of Rb protein.

**Keywords:** gossypol; mammary cancer; fibrosarcoma; antiproliferation; cell cycle; Rb protein; cyclin D1; phosphorylation of Rb protein

Gossypol, a polyphenolic compound extracted from cotton seeds, has long been recognized as an anti-fertility agent and, more recently, it has been demonstrated to inhibit the growth of various carcinoma cell lines in vitro (Floridi et al, 1983; Joseph et al, 1983; Haspel et al, 1984; Tuszynski and Cossu, 1984; Band et al, 1989; Benz et al, 1990; Jaroszewski et al, 1990) and in vivo (Wu et al, 1989; Rao et al, 1985) including oestrogen-sensitive (MCF-7 and MCF-7 Adr) and -insensitive (MDA-MB-231) human mammary cancer cells (Hu et al, 1993; Gilbert et al, 1995). Clinically, gossypol has been efficacious in the treatment of metastatic adrenal cancer (Flack et al, 1993), holding promise as an anti-tumour agent.

Numerous biochemical studies have been conducted to elucidate the mechanisms by which gossypol exerts its antiproliferative effects (Rosenberg et al, 1986; Adlakha et al, 1989). Data are limited and understanding of gossypol's influence on cell cycle control of DNA synthesis and antimitogenic activity is incomplete (Wang and Rao, 1984; Thomas et al, 1991). We wished to determine whether gossypol could induce changes in the expression of cell cycle regulatory proteins, such as the retinoblastoma (*Rb*) gene product (pRb) and cyclin D1, in human mammary cancer cells, a tumour type associated with mutation of the *Rb* gene (Lee et al, 1988; T'Ang et al, 1988; Varley et al, 1989) as well as over-expression and amplification of cyclin D1 (Buckley et al, 1993; Keyomarsi and Pardee, 1993). In addition, we studied the influence of gossypol on the phosphorylation of Rb protein. For this purpose we used the human mammary cancer cell line MCF-7, which has oestrogen and progesterone receptors, as do certain human cancers in vivo. Additionally, we wished to study gossypol effects on cell cycle phases in MCF-7 cells. Furthermore, to determine the importance of gossypol inhibition on cyclin D1 and Rb protein in its antiproliferative effect, we performed similar studies in human fibrosarcoma cells that overexpress cyclin D1.

*Received 9 April 1996*
*Revised 20 November 1996*
*Accepted 14 January 1997*

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## MATERIALS AND METHODS

### Cell culture

MCF-7 human mammary cancer cells and HT 1060 human fibrosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

The HT 1080 cells were maintained in log-phase growth in RPMI medium (Media Preparation Core Facility, Sloan-Kettering Institute) supplemented with 10% FCS (Sigma, St Louis, MO, USA). Transfection of a cyclin D1-expressing plasmid was carried out as previously described (Hochhauser et al, 1996). Relative expression of cyclin D1 mRNA ratios were 14.4 and 0.48 for the high- and low-expression clones, whereas the comparable cyclin D1 expression ratios were 2.88 and 0.9 as compared with the vector-only-transfected control (Hochhauser et al, 1996).

Racemic gossypol (Sigma) dissolved in dimethylsulphoxide (DMSO) was added to culture medium samples [Dulbecco's modified Eagle medium (DMEM) (Ham's F12/DME, 2:1, v/v) supplemented with 10% fetal calf serum (FCS), 100 μg $ml^{-1}$ streptomycin, 100 U $ml^{-1}$ penicillin and 2 mM glutamine] and incubated at 37°C in a 5% carbon dioxide/95% air atmosphere for 24 h. In order to minimize gossypol oxidation, reduced glutathione was added to the culture medium (2 mM).

### [³H]Thymidine incorporation assay

MCF-7 human mammary cancer and HT 1080 human fibrosarcoma cells were inoculated individually in six-well dishes (9.6 $cm^2$ area) in a volume of 2 ml of DMEM supplemented with 10% FCS at a density of $5 \times 10^4$ cells per well. After 2 days, gossypol dissolved in DMSO was added to the culture medium. Incubations with gossypol were carried out for various time intervals at 37°C. Before cell harvest, cells were labelled with [³H]thymidine (20 µCi per well) at 37°C for 3 h and washed three times with Hanks' balanced salt solution (HBSS). Cells were solubilized with 0.5% SDS (w/v) at 37°C for 10 min.

To cell lysates, 10% trichloroacetic acid (TCA) (v/v) was added and incubation continued for 30 min on ice. TCA-precipitated samples were filtered using glass fibre filters (Enzo Diagnostics, Syosset, NY, USA) to separate bound and free radioactivity. Filters were then washed three times with ice-cold 10% TCA (v/v). Radioactivity retained on the filters was determined with a scintillation counter. The radioactivity of each sample was normalized by protein concentration determined by the A562 microbicinchoninic acid protein assay (Pierce Chemical, Rockford, IL, USA). All colorimetric procedures were carried out with a Gilford model 260 spectrophotometer.

### Cell cycle analysis

MCF-7 cells treated with various concentrations of gossypol were trypsinized. Cell suspensions were centrifuged (1000 r.p.m., 10 min) and then washed twice with $Ca^{2+}/Mg^{2+}$-free HBSS to remove excess trypsin. After the final wash, cell pellets were resuspended in 1 ml of HBSS buffer. Cells were then fixed and permeabilized with 70% (v/v) ethanol at 4°C overnight. Next day, cell pellets were prepared by centrifugation at 1000 r.p.m. for 10 min. Cell pellets were resuspended in HBSS buffer containing 50 µg $ml^{-1}$ propidium iodide. Incubation continued for 1 h at room temperature. Cells were filtered through nylon mesh (41 µm) (Spectrum, Houston, TX, USA).

DNA content was analysed on an Epics Profile Cytometer. Propidium iodide-stained nuclei were excited with a 488-nm air-cooled argon laser, and fluorescence emission greater than 680 nm was recorded on a linear scale. A minimum of 20 000 nuclei were counted per sample. Doublets and clumps were excluded from the analysis by gating on a bivariate distribution of the peak fluorescence signal.

### Western blot analysis

After incubation for periods up to 3 days with different concentrations of gossypol, MCF-7 or cyclin D1-transfected HT 1080 human fibrosarcoma cells were washed twice with ice-cold HBSS and then lysed at 4°C with extraction buffer [20 mM Hepes buffer (pH 7.2), 1% Triton-X 100 (v/v), 10% glycerol (v/v), 2 mM sodium fluoride, 1 mM sodium orthovanadate, 50 µg $ml^{-1}$ leupeptin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF). Cell lysates were clarified by centrifugation at 15 000 r.p.m. at 4°C for 30 min. Supernatants containing equal amounts of protein in each lane were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE)

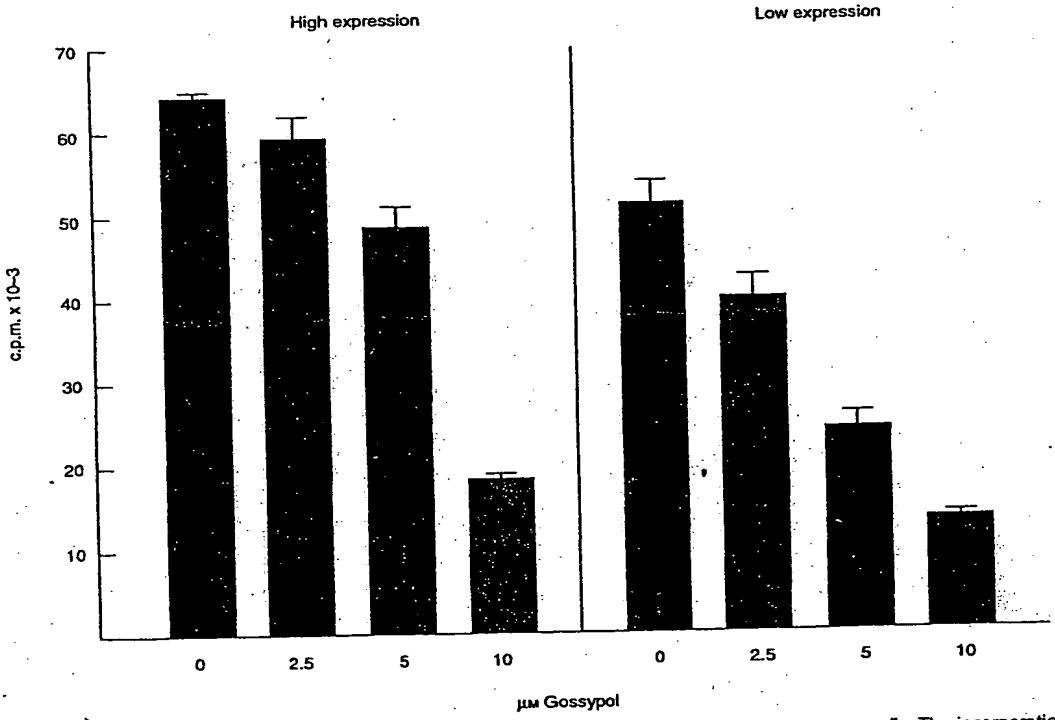


Figure 1 Effect of gossypol concentration on antiproliferative action in high and low cyclin D1-expressing fibrosarcoma cells. The incorporation of [³H]thymidine into DNA was determined in fibrosarcoma cells after addition of gossypol (2.5–10 µM) or 95% (v/v) DMSO (final concentration < 0.2%, v/v). Results are expressed as c.p.m. [³H]thymidine per unit ($OD_{260}$) of protein and represent the means ± s.d. of triplicate wells. Data presented are representative of three similar experiments

Table 1 Cell cycle distribution of MCF-7 cells after gossypol treatment

| Gossypol (μM) | Cell cycle phases (% of cells) $G_0/G_1$ | S | $G_2$ | Mitotic index |
|---|---|---|---|---|
| *After 24 h treatment* | | | | |
| Control | 63.3 | 26.9 | 9.8 | 0.579 |
| 0.1 | 68.8 | 23.4 | 7.7 | 0.452 |
| 1 | 69.5 | 23.5 | 7.8 | 0.450 |
| 2.5 | 71.4 | 20.5 | 8.2 | 0.401 |
| 5 | 71.2 | 24.3 | 4.5 | 0.404 |
| 7.5 | 78.2 | 17.8 | 4.8 | 0.289 |
| 10 | 78.8 | 16.7 | 5.2 | 0.277 |
| *After 48 h of treatment* | | | | |
| Control | 81.4 | 12.9 | 5.7 | 0.228 |
| 1 | 78.6 | 15.5 | 5.9 | 0.272 |
| 2.5 | 80.5 | 10.8 | 8.7 | 0.242 |
| 5 | 87.95 | 6.15 | 5.9 | 0.136 |
| 7.5 | 91.1 | 3.65 | 5.3 | 0.097 |
| 10 | 88.8 | 6.05 | 5.15 | 0.126 |

using a Bio-Rad miniprotein II electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis at a constant 150 V for about 1 h, proteins in the gel were transferred to a nitrocellulose membrane (Bio-Rad) by electroblot transfer at 100 V for 2 h at 4°C in a transfer buffer (pH 8.3) containing 20% methanol (v/v), 150 mM glycine and 20 mM Tris, using a Bio-Rad minitransblot electrophoretic transfer apparatus. Rainbow-coloured protein molecular weight standards obtained from Amersham were used for the estimation of molecular size. Membranes with transferred proteins were treated with blocking solution [1 × TBS (Tris-buffered saline), fraction V 3% bovine serum albumin (BSA), 0.2% Tween 20 (v/v)] for 1 h at room temperature and washed with 1 × TBS buffer for 20 min. Purified mouse anti-human Rb gene product monoclonal antibody (Pharmingen, San Diego, CA, USA) (1 μg $ml^{-1}$), or rabbit anti-human cyclin D1 polyclonal antibody (Upstate Biotechnology Inc, Saranac Lake, NY, USA) (1 μg) in blocking solution was then added to the membranes and incubated overnight at 4°C. Similar Western blot analyses were performed with control proteins and their corresponding antisera, i.e. cdk4, p21, actin and vinculin.

On the following day the nitrocellulose membranes were washed with 1 × TBS for 20 min and incubated with horseradish peroxidase conjugated either with anti-mouse or anti-rabbit IgG for 1 h at room temperature. Secondary antibody was at a concentration of 1:1000 dilution. After reaction, membranes were washed and developed by chemiluminescence (ECL) (Amersham) (Whitehead et al, 1979), and exposed to XAR5 film (Kodak).

The monoclonal antibody for pRB was obtained from Pharmingen (cat. no. 14001A). Cell extracts were prepared as in the manufacturer's instructions (Santa Cruz Biotechnology). Protein concentrations were estimated by the Bradford assay as described. Immunoblots were prepared as in the manufacturer's instructions (Santa Cruz Biotechnology). Total protein (100 μg) was loaded for each sample after addition of SDS polyacrylamide sample buffer and boiling for 5 min. Protein loading was visualized by Poinceau staining. Samples were electrophoresed on 7% polyacrylamide/SDS gels. Nitrocellulose membranes (Optitran, Schleicher & Schuell) were incubated with the enhanced chemiluminescence system as described in the manufacturer's instructions (Amersham) and exposed to film.

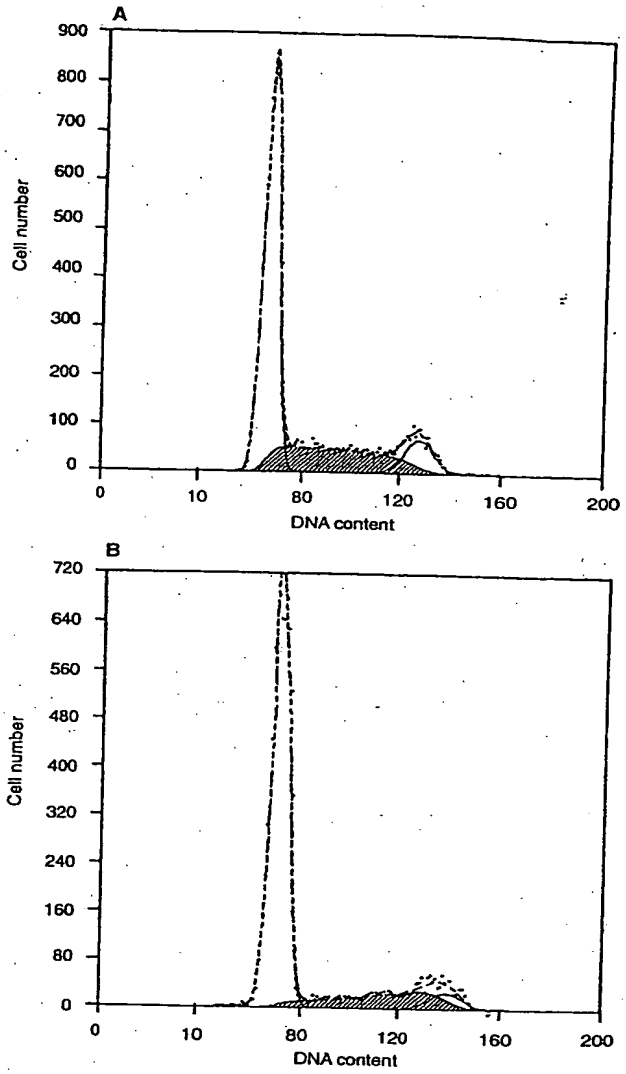


Figure 2 Representative DNA histograms of (A) untreated MCF-7 cells. Cell cycle data: % $G_1$ = 63.3, % $G_2$ = 9.8, % S = 26.9, $G_2/G_1$ = 1.926; chi-square = 1.4. (B) MCF-7 cells treated with 10 μM gossypol after 24 h (flow cytometry). Cell cycle data: % $G_1$ = 78.0, % $G_2$ = 5.2, % S = 16.7 $G_2/G_1$ = 1.960; chi-square = 2.9

## In vitro cyclin D1 kinase assay

MCF-7 cells ($10^6$ cells per 100 mm dish) treated with or without 10 μM gossypol for 24 h were lysed in 0.3 ml of lysis buffer containing 50 mM Tris (pH 8.0), 120 mM sodium chloride, 50 mM sodium fluoride, 0.1 mM sodium vanadate, 2 mM EDTA, 10 μg $ml^{-1}$ each of chymostatin, leupeptin, antipain and pepstatin A; 2 μg $ml^{-1}$ 4-(2-aminoethyl)benzenesulphonyl fluoride and 0.4% Nonidet P-40. The extracts were clarified by centrifugation at 14 000 r.p.m. for 15 min at 4°C. Lysates were incubated for 1.5 h at 4°C with polyclonal antibody against cyclin D1. Immune complexes were collected using 20 μl of protein A–Sepharose and washed three times with 1 ml of lysis buffer and once with 1 ml of kinase buffer containing 20 mM Tris (pH 7.5)–10 mM magnesium chloride. Histone H1 kinase assay was performed on a bead. The

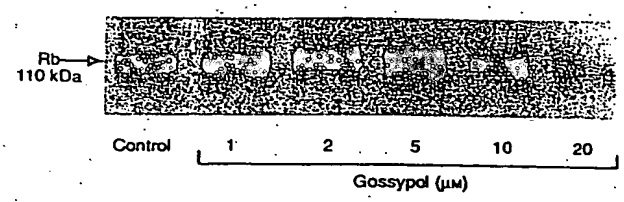


Figure 3 Western blot analysis of the effect of gossypol on the expression of Rb protein in MCF-7 cells. Cells were treated with the indicated gossypol concentrations for 24 h. Protein from cell lysates (20 μg) was loaded into each lane for Western blot using mouse anti-human Rb monoclonal antibody (1 μg ml⁻¹). Detection of Rb protein was done by the ECL method

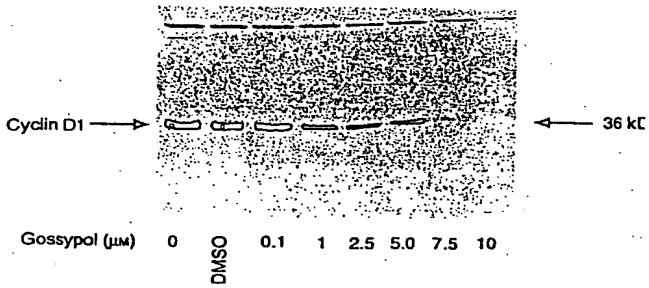


Figure 4 Western blot analysis of the effect of gossypol on the expression of cyclin D1 in MCF-7 cells. Cells were treated with the indicated gossypol concentrations for 24 h. A 100-μg aliquot of protein from cell lysates (100 μg) was loaded into each lane for Western blot using polyclonal rabbit anti-human cyclin D1 polyclonal antibody (1 μg ml⁻¹). Detection of cyclin D1 was done by the ECL method

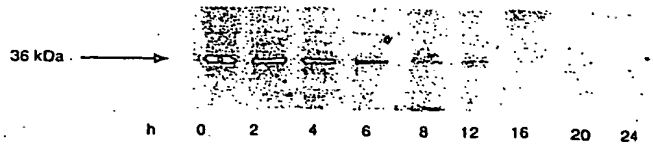


Figure 5 Time course of gossypol effect on the expression of cyclin D1 in MCF-7 cells. Cells were treated with 10 μM gossypol for the indicated periods of time. Protein (100 μg) from cell lysates was loaded into each lane for Western blot analysis using rabbit anti-human cyclin D1 polyclonal antibody (1 μg ml⁻¹). Detection of cyclin D1 was done by the ECL method

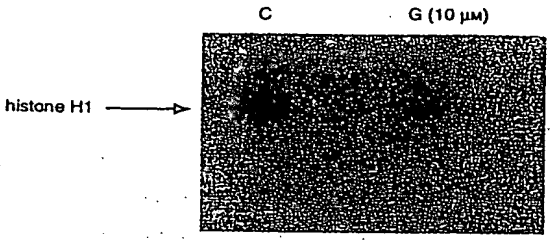


Figure 6 Effect of gossypol on histone H1 kinase. MCF-7 cells were treated with or without 10 μM gossypol for 24 h. Total cell lysates (50 μg) were immunoprecipitated with polyclonal anti-human cyclin D1 antibody conjugated with protein A-Sepharose. Immunoprecipitated samples were assayed for histone H1 kinase activity by incubation of [³²P-γ]ATP with histone H1 as detailed in Materials and methods. Protein from cell lysates (100 μg) was loaded into each lane and was subjected to SDS-PAGE and [³²P]histone H1 identified by radioautography

beads were mixed with 15 μl of a kinase reaction mix containing 2 μg of histone H1 and [³²P]ATP. After 30 min at 30°C, 25 μl of 2 × SDS-PAGE buffer was added and 20 μl was analysed by SDS-PAGE and autoradiography.

## RESULTS

### Antiproliferation

As in other systems, the antiproliferative effects of gossypol have been established with [³H]thymidine uptake and cell stage analyses (Wang and Rowe, 1984; Thomas et al, 1991). We employed these techniques to validate their applicability to MCF-7 human mammary cancer cells and HT1080 fibrosarcoma cells transfected with cyclin D1.

We assessed the antiproliferative effects of gossypol by counting the number of viable MCF-7 cells (trypan blue exclusion). Gossypol inhibited growth of MCF-7 human mammary cancer cells in a dose-dependent manner with an estimated $IC_{50}$ of 3 μM over a 3-day incubation period (data not shown). In addition, the dose dependence and the kinetics of inhibition of [³H]thymidine incorporation into DNA were determined. In the MCF-7 cells the pattern of anti-mitogenesis was dose and time related (data not shown). Six hours after the addition of the drug, all gossypol concentrations produced a significant decrease in thymidine uptake, with 10 μM gossypol causing a 50% reduction in thymidine incorporation. Gossypol concentrations of 7.5 and 5 μM attained 50% reduction in thymidine incorporation after 11 and 16 h respectively.

From [³H]thymidine uptake, we determined the antiproliferative effects of gossypol in both high and low cyclin D1-transfected fibrosarcoma cells. After exposure to increasing concentrations (2.5–10 μM) of gossypol for 24 h, we observed a progressive decrease in ³H incorporation in both high and low cyclin D1-expressing fibrosarcoma cells with $IC_{50}$ values of 8 μM and 4 μM respectively (Figure 1).

### Effects of gossypol on the cell cycle phases in MCF-7 human mammary cancer cells

We determined whether the antimitogenic effects of gossypol in MCF-7 cells were cell cycle related. The effects of different gossypol concentrations on cell cycle phases were studied with a fluorescence-activated cell sorter (FACS) at 24 and 48 h (Table 1). After 24 h, and more noticeably after 48 h, exposure to gossypol was associated with a significant decrease in the proportion of cells in S phase, when replication of DNA occurs (12.9% for untreated cells as compared with 3.65% for the 7.5 μM gossypol-treated cells). In addition, the percentage of cells in the $G_1$ pre-mitotic stage was progressively raised with increasing gossypol concentrations (78.8% for 10 μM gossypol-treated cells as compared with 63.3% for control). There was a decrease in the proportion of cells in $G_2$. Figure 2 shows representative DNA histograms of MCF-7 cells in culture medium for 24 h: (a) untreated cells; (b) 10 μM gossypol-treated cells. These results support the conclusion that gossypol reduces the mitotic index ($MI = S+G_2/M/G_1$) in MCF-7 cells by blocking cells in the $G_1$ phase, as is evidenced by a dose-dependent increase in cell percentages in these phases.

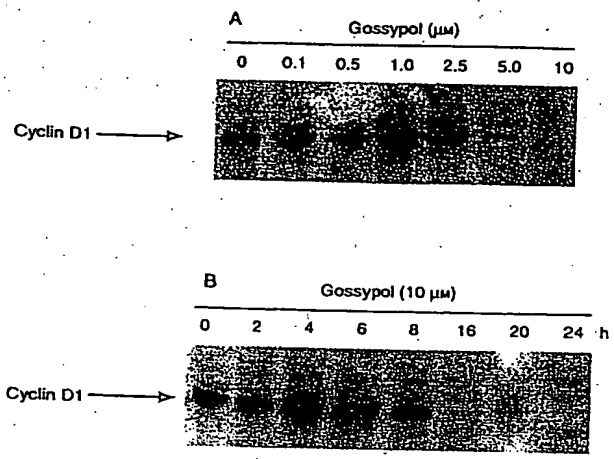


Figure 7 Western blot analysis of cyclin D1 expression with varying concentrations of gossypol (0.1–10 μM) (Figure 8A) and time (at 10 μM) gossypol (Figure 8B) on high-expression cyclin D1 human fibrosarcoma cells

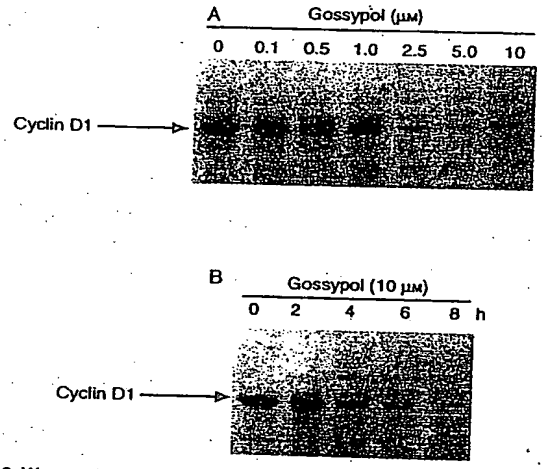


Figure 8 Western blot analysis of Rb protein expression with varying concentrations of gossypol (0.1–10 μM) (Figure 9A) and time (at 10 μM) (Figure 9B) on low-expression cyclin D1 human fibrosarcoma cells

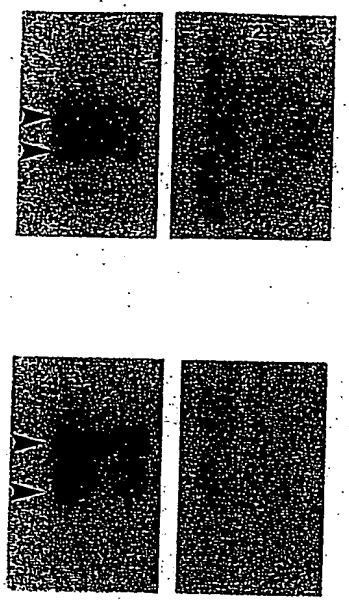

Figure 9 Western blot of total cell extracts probed with a monoclonal antibody for pRb. Samples were electrophoresed on a 7% polyacrylamide/SDS gel. The upper and lower arrows denote phosphorylated and underphosphorylated pRb respectively. Lane 1, HT1080 cells transfected with neo; lane 2, HT1080 cells expressing cyclin D1; lane 3, HT 1080 cells; lane 4, HT1080 cells expressing cyclin D1 exposed to 7.5 μM gossypol. Lane 1, HT1080 cells expressing low levels of cyclin D1; lane 2, HT1080 cells expressing low levels of cyclin D1 exposed to 7.5 μM gossypol; lane 3, HT1080 cells expressing high levels of cyclin D; lane 4, HT1080 cells expressing high levels of cyclin D exposed to 7.5 μM gossypol

## Effects of gossypol on cell cycle related proteins

### *MCF-7 human mammary cancer cells*

As pRb is an important cell cycle protein governing transition from $G_1$ to S phase we investigated whether the activity of gossypol could be mediated through expression of tumour-suppressor genes. We determined whether the expression of pRb was changed following exposure to gossypol. Western blot analysis using mouse anti-human Rb monoclonal antibody demonstrated that, after 24 h, gossypol decreased expression levels of Rb protein in MCF-7 cells in a dose-dependent manner (Figure 3). This suggests that gossypol might act, in part, by decreasing Rb protein in MCF-7 cells. The kinetics of the gossypol effect on Rb protein expression indicate that 10 μM gossypol decreased Rb protein levels as early as 8 h and almost completely at 16 h (data not shown). Western blot analysis of MCF-7 cell lysates treated with gossypol revealed only a single band in control and lower concentrations (1 and 2 μM) of gossypol. At concentrations of 5 μM and higher a second, more rapidly migrating, band appeared with disappearance of the more slowly migrating band (Figure 3). This is consistent with the slower moving band representing phosphorylated Rb protein and the more rapidly moving band being non-phosphorylated Rb.

Whereas the growth-suppressing activity of Rb is regulated by its phosphorylation state, which in turn is regulated by cyclin D1/Cdk4 complexes in other mammalian cells, it was of interest to determine whether gossypol also affects the expression of these proteins in MCF-7 cells. We have found that gossypol also decreased cyclin D1 protein levels in MCF-7 cells in a dose-dependent manner (Figure 4). The effect of 10 μM gossypol on cyclin D1 was apparent as early as 6 h and almost completely at 16 h (Figure 5). Gossypol (10 μM) decreased cyclin D1-associated kinase activity on histone H1 as a substrate in MCF-7 cells after 24 h of treatment (Figure 6). Although gossypol at the highest concentration tested (10 μM) produced a 50% antiproliferative effect, gossypol (10 μM) over 24 h had no effect on expression of Cdk4, actin, vinculin or p21 (data not shown). Thus, the effects on cyclin D, Rb and histone H1 kinase would appear not to be an experimental artifact due to cell loss.

### HT 1080 cyclin D1 overexpressing human fibrosarcoma cells

In view of the effects of gossypol on cyclin D1 expression in MCF-7 cells, it was of interest to determine the influence of gossypol in cells expressing high levels of cyclin D1. Incubation of high-expression cyclin D1 human fibrosarcoma cells with gossypol led to a decrease in cyclin D1 expression with half-maximal responses between 2.5 and 5 μM (at 24 h) and approximately 12 h (at 10 μM) (Figure 7A and 7B). With low-expression cyclin D1 human fibrosarcoma cells, there was a decrease in cyclin D1 expression with half-maximal responses at approximately 2 μM (at 24 h) and approximately 4 h (at 10 μM) (Figure 8A and B).

To investigate whether overexpression of cyclin D1 would modulate the antiproliferative effect of gossypol, we exposed HT1080 cells transfected with cyclin D1 to drug. These cells express increased amounts of cyclin D1 and show an increase in the number of cells in S and $G_2$ phases. There is consequently an increased proportion of phosphorylated pRb in cells over-expressing cyclin D1 on immunoblotting. The HT1080 cell line expressing the neo vector only and a transfectant with high levels of cyclin D1 were exposed to varying doses of gossypol (Figure 1). The results indicated an $IC_{50}$ of 4 μM and 8 μM respectively.

Immunoblotting of HT1080 cells expressing the neo vector and a transfectant expressing high levels of cyclin D1 was carried out (Figure 9). As previously noted, there is an increase in the proportion of phosphorylated pRb in the cell line expressing high levels of cyclin D1. Exposure to gossypol reduced expression of pRb in both cell lines. However, even after exposure to 7.5 μM gossypol, pRb was exclusively in the unphosphorylated state in the neo-expressing cells, whereas phosphorylated pRb was detectable in the clone expressing high levels of cyclin D1. This suggests that resistance to gossypol-induced growth arrest in the line expressing high levels of cyclin D1 may be due to the increase in the proportion of phosphorylated pRb in this line compared with the parental cell line.

## DISCUSSION

Numerous biochemical effects of gossypol have been described, such as uncoupling of oxidative phosphorylation and inhibition of many membrane-associated enzymes (Lee et al, 1982; Bugeja et al, 1988; Nakamura et al, 1988). Indeed, in our earlier studies of the effects of gossypol on human erythrocyte function, we noted that 10 μM gossypol inhibited inorganic anion exchange and interaction with band 3 without effect on eight other membrane functions (Haspel et al, 1985). However, it has been difficult to determine the specific site and mechanism of action or link these actions to the tumoricidal effects of gossypol in vitro.

To elucidate other molecular mechanisms that could mediate gossypol's antiproliferative effects (data not shown), we first assessed the overall effect of gossypol on the cell cycle of MCF-7 cells. Our data on cell cycle analysis in non-synchronized populations of MCF-7 cells suggest that gossypol arrests cells in $G_1$/S, in agreement with other studies demonstrating that gossypol inhibits growth in vitro by reducing the growth fraction (Wang and Rao, 1984; Thomas et al, 1991). As gossypol specifically acts in the $G_1$ phase to prevent cells from entering S phase, it was of interest to determine whether gossypol could affect cell cycle-regulated proteins, in particular Rb and cyclin D1 proteins, which are critical for $G_1$ to S progression. Rb protein is known to be crucially involved in cellular growth regulation and exists as hypo- and hyperphosphorylated forms, its phosphorylation status being highly cell cycle phase dependent (Cooper & Whyte, 1989). The unphosphorylated form of the Rb protein is found in quiescent and $G_1$ phase cells, restricting $G_1$ to S progression by an interaction with the E2F transcription factor (Chellappan et al, 1991). It is a target of complex formation with several oncoproteins, e.g. E7, known to have an immortalizing effect on infected cells (Green, 1989), an inactivation mechanism functionally similar to the Rb protein phosphorylation or to its loss by gene mutation or deletion, resulting in unregulated cell proliferation. Introduction of the Rb gene into cancer cells lacking a functional endogenous Rb gene has been found to reverse their transformed phenotype and tumorigenicity, a finding providing conclusive evidence of its tumour-suppressing activity (Huang et al, 1988).

We found that the expression levels of the Rb and pRb proteins decreased in response to gossypol treatment in MCF-7 cells, a cancer cell line that predominantly expresses the phosphorylated form of the Rb protein (Lee et al, 1988). In SDS-PAGE gels in which non-phosphorylated was separated from phosphorylated Rb protein, we noted a greater decrease in the more slowly migrating pRb band. This is consistent with a decrease in phosphorylated Rb due to inhibition of phosphorylation. There may also be inhibition of Rb protein expression after gossypol treatment (Figure 3). Whether gossypol affects Rb protein expression at the transcriptional and/or translational as well as post-translational level remains to be determined.

We have also demonstrated that gossypol decreases the expression of cyclin D1 protein in MCF-7 cells. Cyclin D1 is considered to be essential for progression through the $G_1$ phase of the cell cycle in a variety of human normal and tumour cells (Baldin et al, 1993; Lukas et al, 1994). It has been shown that cyclin D1 associates with Cdk4 during the $G_1$ phase in synchronized cells (Kato et al, 1993). The cyclin D1–Cdk4 complex assembled in a subcellular assay or as a result of the coexpression of cyclin D1 and Cdk4 in intact insect cells phosphorylates the Rb protein in vitro (Matsushime et al, 1992; Kato et al, 1993). It has been suggested, therefore, that cyclin D1 functions by inactivating the inhibitory effects of the Rb protein on cell cycle progression (Jiang et al, 1993). Gossypol treatment did not affect Cdk4 levels but inhibited cyclin D1 expression. This may account for the observed reduction in cyclin D1-associated kinase activity on histone H1 in MCF-7 cells (Figure 6). Gossypol, through its ability to decrease the concentration of cyclin D1 may effectively decrease the amount of phosphorylated Rb and, thus, arrest cells in $G_1$. The correspondence of the gossypol effects on both Rb and cyclin D1 as reflected in similar kinetics (Figure 5) and concentration (Figures 3 and 4) is consistent with cyclic D1 in association with Cdk4 catalysing the phosphorylation of Rb. The effects of gossypol on other cyclins and Cdks required for entry into S phase, such as cyclin E, Cdk2 and Cdk5, remain to be studied. To confirm the role of cyclin D1 in mediating the effect of gossypol, we exposed HT1080 cells transfected with cyclin D1 to gossypol. These cells have been demonstrated to have increased phosphorylation of pRb. Gossypol has a lesser effect on proliferation in transfectants with high cyclin D1. Furthermore, although these cells also show a reduction in pRb expression after exposure to gossypol, phosphorylated pRb is detectable in cells with high cyclin D1.

Recently, small protein inhibitors of cyclin–Cdk (Ckis) have been shown to play an important role in regulating the activity of cyclin-dependent kinases. In mammals, p16, p21 and p27 have been shown to inhibit cyclin D1–Cdk4 (Toyoshima and Hunter,

1994). In a preliminary study, we investigated whether gossypol-induced $G_1$ arrest could be mediated by changes in the expression of p21, a protein known to interact with several cyclin–Cdks in vivo (Harper et al, 1993). Although p21 levels did not change after gossypol treatment (data not shown), the effects of gossypol on other Ckis remain to be determined.

Our in vitro assays support, but do not prove, an association between the cell cycle-modulating activity of gossypol and its antiproliferative effects. There is a similar course of gossypol inhibition of thymidine incorporation into DNA as of gossypol inhibition of cyclin D1 and Rb protein expression and phosphorylation in MCF-7 cells.

If the in vitro changes observed in the expression of Rb and cyclin D1 proteins account for the antiproliferative properties of gossypol, this may prove conceptually and therapeutically important through cell cycle regulation (reregulation). In addition, whether the observed changes in cyclin D1 protein expression and Rb protein expression and phosphorylation represent the essential feature associated with both the anti-tumour and contraceptive properties of gossypol remain to be established.

## ACKNOWLEDGEMENTS

This work was supported in part by a Merck Sharp & Dohme International Fellowship in Clinical Pharmacology, 1993 (to ML), Grant DK 41931 from the National Institutes of Health (to MS), GA PS 9411 from The Rockefeller Foundation and Hoffmann-LaRoche, Inc. (to MMR) and NIH CA 09512 from the Clinical Scholars Biomedical Research Training Program (to BT). The authors are grateful to Wayne Douglas and Jenny Fu for excellent technical contributions and to Sigrid Whaley and Ruth Garcia for assistance in the preparation of the manuscript.

## ABBREVIATIONS

BSA, bovine serum albumin; Cdk, cyclin-dependent kinase; Cki, cyclin-dependent kinase inhibitory protein; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulphoxide; ECL, enhanced chemiluminescence; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PMSF, phenylmethylsulphonyl fluoride; Rb, retinoblastoma; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TCA, trichloroacetic acid.

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0021-972X/93/7604-1019$03.00/0
Journal of Clinical Endocrinology and Metabolism
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Vol. 76, No. 4
Printed in U.S.A.

# Oral Gossypol in the Treatment of Metastatic Adrenal Cancer*

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ABSTRACT

Medical treatment of metastatic adrenal cancer is largely unsuccessful and has considerable toxicity. We previously demonstrated the activity of the plant toxin gossypol against human adrenal cancers in nude mice. We therefore examined the efficacy and toxicity of oral gossypol as a treatment for adrenal cancer in humans. Twenty-one patients with metastatic adrenal cancer received oral gossypol at doses of 30–70 mg/day. Patients were monitored for side effects of gossypol, changes in hormone secretion, and tumor response. Eighteen patients completed at least 6 weeks of gossypol treatment. Three of these patients, whose tumors were refractory to other chemotherapeutic agents, had partial tumor responses (≥50% decrease in tumor volume) that lasted from several months to over 1 yr. One patient had a minor response followed by resection of her remaining disease. 1 patient had stable disease, and 13 patients had disease progression. Three patients died of their disease without receiving sufficient gossypol to achieve detectable drug levels, and were eliminated from the final analysis. The side effects of gossypol were generally well tolerated; the only serious side effect was abdominal ileus that resolved when the drug was temporarily withheld and restarted at a lower dose. We conclude that oral gossypol can be used relatively safely on an outpatient basis for the treatment of metastatic adrenal cancer. The response rate is similar to the other agents currently available for adrenal cancer, and responses were seen in patients who had failed other chemotherapeutic regimens. This study provides the first indication that gossypol may have activity against cancer in humans, suggesting the need for further investigation of gossypol as an antitumor agent. (*J Clin Endocrinol Metab* **76:** 1019–1024, 1993)

ADRENAL cancer is a rare, fatal malignancy for which medical therapy is largely unsuccessful. Ortho-para DDT (mitotane) and other chemotherapeutic regimens have partial response rates of only 10–20%, serious side effects, and do not prolong survival (1, 2). Thus, new medical therapies for metastatic adrenal cancer are needed. We have previously shown that gossypol, a spermatotoxin derived from crude cottonseed oil, inhibits the growth of human adrenocortical tumors in nude mice (3). Other animal studies demonstrate the activity of ip gossypol against Ehrlich ascites tumor and mouse mammary carcinoma, but with considerable drug toxicity (4, 5). With oral gossypol, however, we did not observe any adverse drug effect and the survival of treated animals was improved over non-treated controls. In addition, large numbers of normal men in China have taken oral gossypol for contraception with relatively few side effects (6, 7). Thus, we examined the efficacy and toxicity of oral gossypol as a treatment for metastatic adrenal cancer in humans.

July 31, 1992.

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* This research is partially supported by The Rockefeller Foundation, NIH Grants GM-07488 and RR-47, and The L. W. Frohlich Charitable Trust.

## Patients and Methods

### *Patient selection*

Twenty-one patients were evaluated at the clinical center of the National Institutes of Health or the New York Hospital-Cornell Medical Center from September 1989 to May 1991. Patients underwent a complete history and physical exam, routine blood studies for electrolytes, liver and kidney function, 24-h urine collections for measurement of urine free cortisol and 17-hydroxysteroid excretion, and computed tomography (CT) and/or magnetic resonance imaging (MRI) of the chest and abdomen. Measurements of plasma estradiol, testosterone, dehydroepiandrosterone-sulfate, and 11-deoxycorticosterone (DOC) were performed as indicated. All patients had adrenal cancer confirmed by examination of their original pathology specimens, and had clearly visible metastatic disease on CT and/or MRI. All had normal electrolytes; all but one had normal renal function, and 11 patients with liver metastases had stable elevations of hepatic transaminases to twice normal levels. Ten patients had secretory tumors: 8 secreting cortisol, 1 secreting testosterone, and 1 secreting DOC. Nineteen patients were previously treated with mitotane at doses of 2–8 g/day for 6–24 months. Mitotane was discontinued due to tumor progression at least 3 months before starting gossypol. Three patients received suramin and/or adriamycin and etoposide (VP-16), in addition to mitotane. Two patients refused prior treatment with available chemotherapeutic agents.

### *Gossypol administration, drug concentrations, hormone levels, and tumor response*

Patients received oral gossypol (racemic gossypol acetic acid, 10 mg compressed tablets, Chinese Academy of Medical Sciences, Beijing, China) beginning at 20 mg/day and increasing by 10 mg/day every 7 days to 30–70 mg/day in divided doses. The first nine patients were

seen daily during the loading phase and every 1–2 weeks thereafter, to monitor side effects, routine blood chemistries, steroid excretion, and gossypol levels. The following patients were seen weekly during the loading phase, and were then seen by their referring physicians, returning every 6 weeks for follow-up. Side effects were monitored by flow sheets, where the presence and severity of side effects were rated from 0 (not present) to 5 (intolerable), and/or patient interviews.

Gossypol concentrations were determined by HPLC on an ESA Coulochem detector (Bedford, MA), in comparison with known gossypol standards (8). Aliquots of 24-h urine collections were sent for measurement of free cortisol by RIA (Smith Kline Bioscience Laboratories, King of Prussia, PA) and 17-hydroxy-steroid excretion by a modification of the Porter-Silber method (Clinical Center Laboratory, NIH, Bethesda, MD). CT and/or MRI scans were done every 6 weeks and tumor volume calculated from the dimensions of each lesion on CT or MRI. More than 50% reduction in tumor volume was considered a partial response, a 10–40% reduction was considered a minor response, and less than 10% change in tumor volume was considered stable disease.

## Results

Eighteen patients completed at least 6 weeks of treatment and had detectable gossypol levels where measurements were available. Three of these 18 patients, who had previously failed other chemotherapeutic regimens, had partial tumor responses that will be described below. One patient had stable disease at the end of 6 weeks, 1 patient had a minor response followed by surgical resection of the all remaining lung nodules, and 13 patients had tumor progression (Table 1). None of the patients had any significant decrease in steroid excretion directly related to gossypol, but 1 patient had a decrease in urine free cortisol coincident with a tumor response. Three patients received gossypol for less than 4 weeks due to the terminal nature of their illness. These patients had undetectable gossypol levels indicating an inadequate trial of gossypol and were thus eliminated from the final analysis.

### *Partial responses*

The first response was in a 36-yr-old man with a cortisol-secreting tumor metastatic to the liver and lung (patient 4, Table 1). The tumor had progressed despite treatment with mitotane (3 g/day for 6 months), suramin (350 mg/M$^2$·day) and a combination of adriamycin and VP-16. Mitotane, suramin, and adriamycin/VP-16 were discontinued 2 yr, 1 yr, and 6 months before starting gossypol, respectively. At the start of treatment, he had shortness of breath, abdominal pain, marked ascites, lower extremity edema, and a urine free cortisol of 1374 nmol/day (normal 30–300 nmol/day). After 4 weeks of oral gossypol (50 mg/day), he had transient right upper quadrant pain and a CT scan showed greater than 90% reduction in the size of the multiple lung metastases, and 80–90% reduction in the volume of multiple hepatic lesions (Fig. 1). He had a marked improvement in exercise tolerance, decreased abdominal pain and ascites, and a urine free cortisol of 132 nmol/day (Fig. 2). While the clinical improvement lasted for 8 months, the plasma gossypol concentrations gradually declined and the lesions began to regrow. Gossypol was discontinued after a total of 9 months of treatment.

The second response was seen in a 54-yr-old woman with a clinically nonsecreting abdominal tumor recurrence (patient 5, Table 1). The tumor failed to respond to treatment with mitotane (2 g/day for 1 yr) and suramin (350 mg/M$^2$/day). Mitotane was discontinued 18 months, and suramin 9 months before starting gossypol. After 4 weeks of oral gossypol (40 mg/day), she had sharp right upper quadrant and

TABLE 1. Clinical characteristics and tumor response in 21 patients with metastatic adrenal cancer on oral gossypol

| Pt | Age/sex | Disease duration (yr) | Hormone secretion | Metastases | Prior treatment | Gossypol dose (mg/day) | Treatment duration (weeks) | Max. gossypol level (ng/mL) | Tumor response |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 55/M | 4 | None | Lung, liver | Mitotane | 30 | 3[a] | <50 | Progression |
| 2 | 17/F | 1.5 | Cortisol | Lung, liver | Mitotane | 40 | 3.5[a] | <50 | Progression |
| 3 | 28/M | 1.5 | Cortisol | Lung, liver, abd | Mitotane | 70 | 6 | 1142 | Progression |
| 4 | 36/M | 3.5 | Cortisol | Lung, liver | Mitotane, Suramin Adriamycin, VP-16 | 50 | 36 | 547 | Partial response |
| 5 | 52/F | 4 | None | Abd, liver | Mitotane, Suramin | 40 | 20 | 465 | Partial response |
| 6 | 34/M | 2 | Cortisol | Lung, liver, abd | Mitotane, Suramin | 50 | 16 | 344 | Progression |
| 7 | 35/M | 1 | Cortisol | Abd, liver | Mitotane | 60 | 12 | 508 | Progression |
| 8 | 23/F | 1 | Cortisol | Lung, abd | Mitotane | 40 | 3[a] | <50 | Progression |
| 9 | 23/F | 1.5 | Cortisol | Lung, liver | Mitotane | 50 | 6 | 131 | Progression |
| 10 | 16/F | 1 | Cortisol | Liver | None | 50 | 6 | 266 | Progression |
| 11 | 23/F | 2.5 | None | Abd, liver | Mitotane | 50 | 6 | N/A | Progression |
| 12 | 30/M | 2 | None | Liver | Mitotane | 50 | 12 | 242 | Progression |
| 13 | 22/F | 3 | None | Liver | Mitotane | 50 | 12 | 175 | Minor response |
| 14 | 48/M | 3 | None | Lung, liver | Mitotane | 50 | 6 | 95 | Stabilization |
| 15 | 67/F | 8 | None | Vertebral bone | Mitotane | 40 | 52 | 83 | Partial response |
| 16 | 82/F | 1 | None | Liver | Mitotane | 40 | 6 | 552 | Progression |
| 17 | 73/F | 2 | Testosterone | Abd, liver | Mitotane | 40 | 6 | 39[b] | Progression |
| 18 | 30/M | 4 | None | Liver | Mitotane | 40 | 6 | 449 | Progression |
| 19 | 51/F | 9 | DOC[c] | Liver | Mitotane | 40 | 6 | 65[b] | Progression |
| 20 | 28/F | 2 | None | Liver, abd | Mitotane | 40 | 6 | N/A | Progression |
| 21 | 52/M | 1.5 | None | Liver, abd | None | 40 | 6 | 559 | Progression |

[a] Patients (Pt) eliminated from the final analysis due to insufficient trial of gossypol.
[b] Maximum (max) gossypol levels not available, values are those obtained during the leading phase.
[c] Deoxycorticosterone.

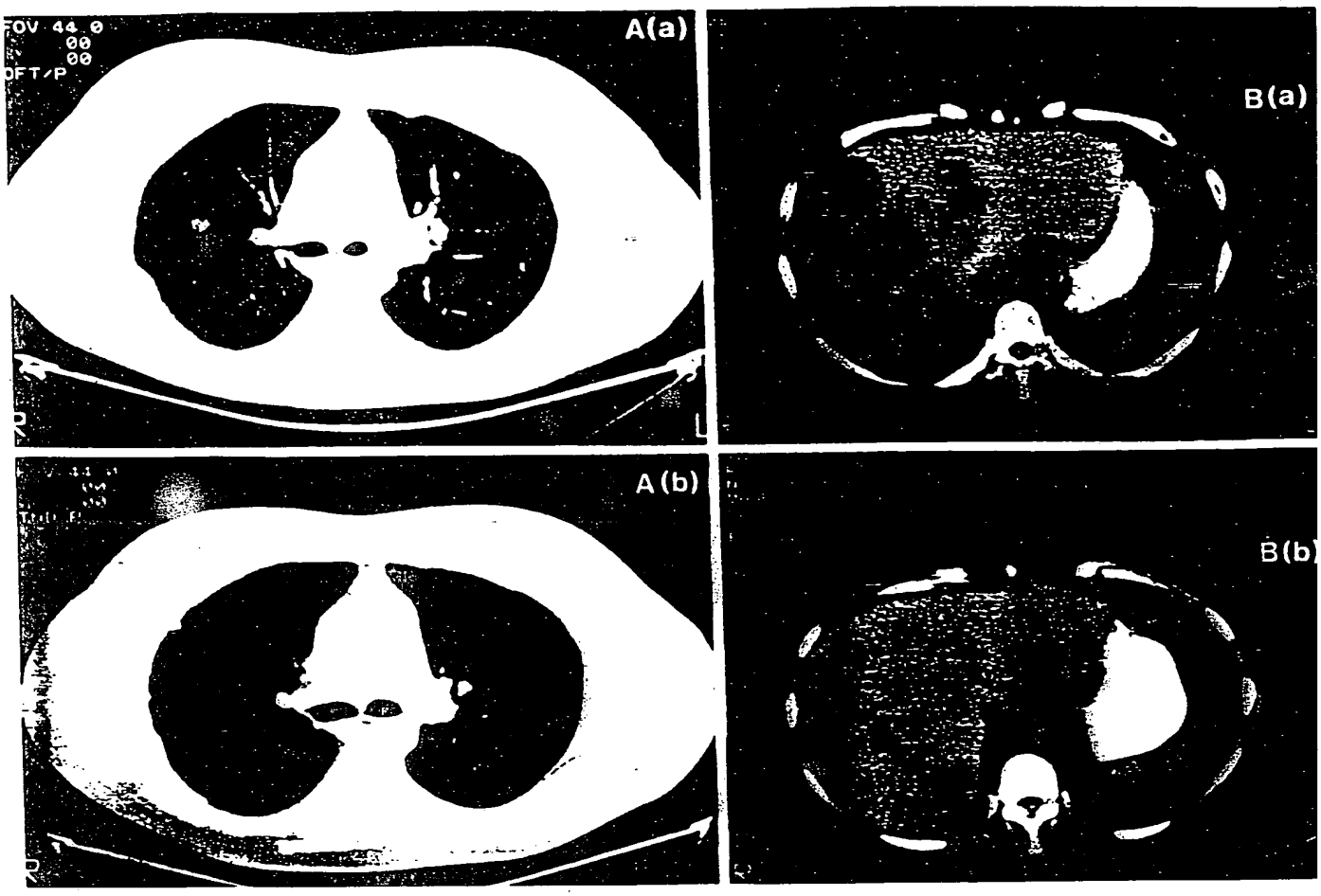


FIG. 1. A. CT of the chest before (a) and during (b) treatment with gossypol (50 mg/day) in a 36-yr-old male with metastatic adrenal cancer CT of the liver before (a) and during (b) gossypol treatment in the same patient.

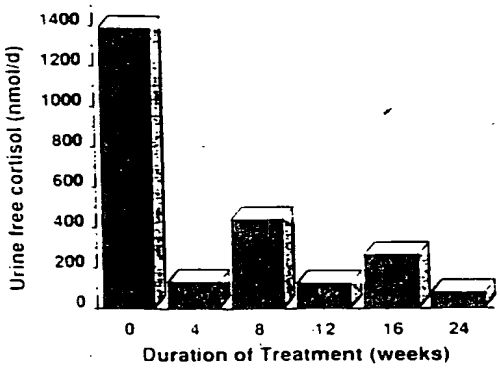


FIG. 2. Urine free cortisol levels during gossypol treatment (50 mg/day) in a 36-yr-old male with metastatic adrenal cancer. The patient had an 80–90% reduction in tumor size at week 4.

right-sided pleuritic pain that resolved over a 1-week period. CT scans after this episode showed complete central necrosis of the abdominal tumor with an 80% reduction in tumor volume, associated with decreased abdominal pain and c tention (Fig. 3A). Despite continuation of gossypol at do of 40–50 mg/day, her plasma gossypol level declined o the next 4 months and gossypol was discontinued due growth of a retroperitoneal lesion.

The third response was seen in a 67-yr-old woman wit nonsecreting T12 paraspinal metastasis causing severe lo back and leg pain (patient 15, Table 1). The tumor previously failed to respond to treatment with mitotan g/day for 1 yr), that was discontinued 1 yr before gossy treatment. After 20 weeks of oral gossypol (40 mg/d there was a 50% reduction in the volume of the parasp lesion associated with a dramatic improvement in her b and leg pain (Fig. 3B). The size of the lesion and the pati symptoms remain stable after 1 yr on oral gossypol at a c of 30 mg/day.

*Gossypol levels and toxicity*

The highest plasma gossypol levels achieved in the tients who received an adequate trial of gossypol rar

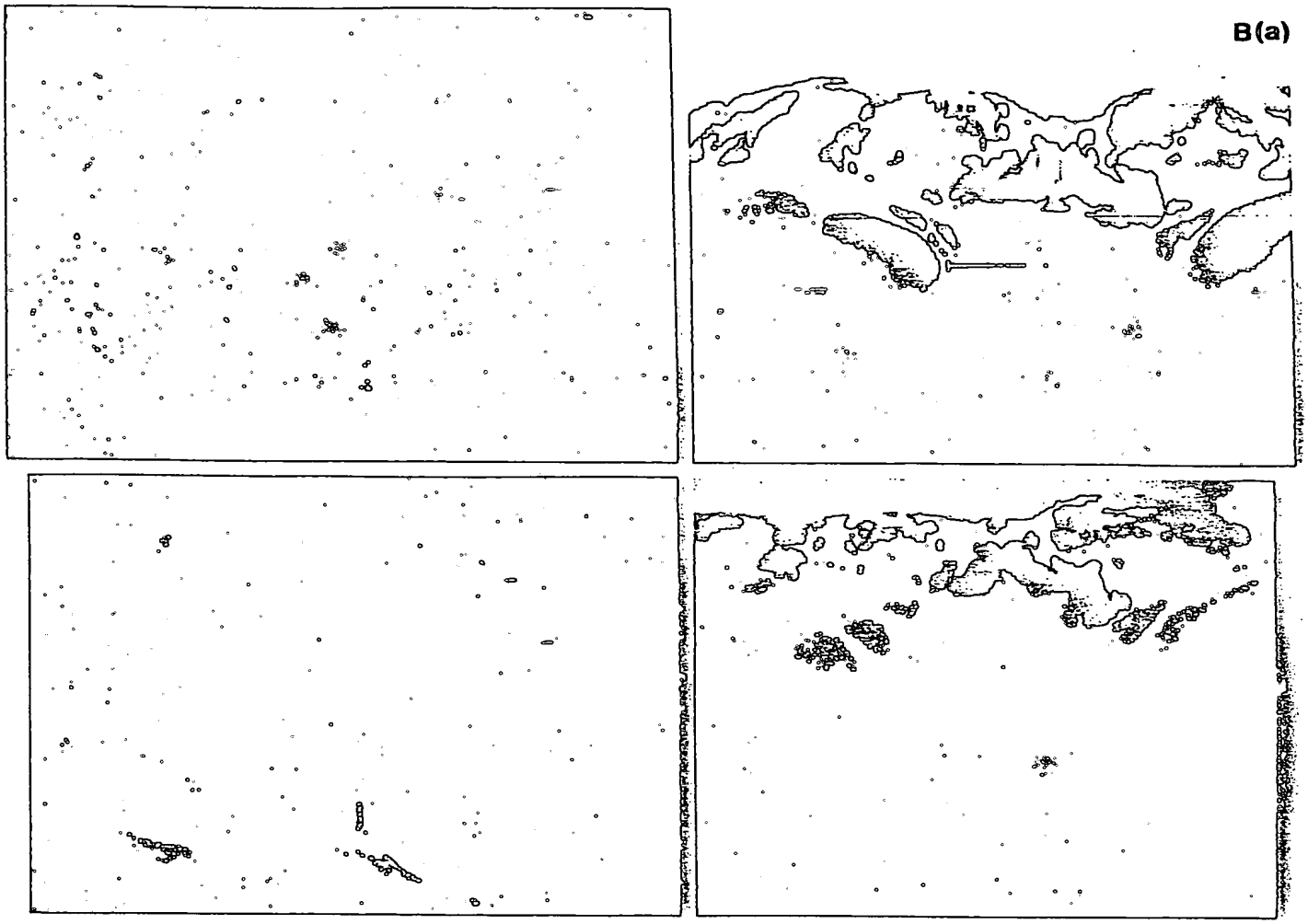


FIG. 3. A. CT of the abdomen before (a) and during (b) treatment with gossypol (40 mg/day) in a 54-yr-old woman with metastatic adrenal cancer. B. MRI of the T12 vertebra before (a) and during (b) treatment with gossypol (40 mg/day) in a 67-yr-old woman with metastatic adrenal cancer.

from 83 to 1,025 ng/dL (Table 1). In general, plasma gossypol levels correlated only roughly with the prescribed gossypol dose. The plasma gossypol levels in patients who had tumor responses were 547, 465, and 83 ng/dL, respectively, at the time of their responses. The plasma gossypol levels in patients who had tumor responses were indistinguishable from the gossypol levels in patients who did not respond. In four patients who were followed after discontinuation of gossypol, the estimated half-time of disappearance was 2.9 +/− 0.9 weeks (Fig. 4).

The side effects of gossypol (and their incidence in the NIH patients) were xerostomia (93%), transient transaminitis (93%), dry skin (71%), fatigue (64%), intermittent nausea (36%), vomiting (21%), transient ileus (21%), and minor hair thinning (14%). In addition, one patient with preexisting gynecomastia had increased size and tenderness of breast tissue while on gossypol therapy. Of the 18 patients who had measurable gossypol levels, no patient had to permanently discontinue gossypol due to its side effects. Four patients had abdominal ileus after receiving gossypol continuously for 3 months at doses of 40 mg/day or more. The plasma gossypol levels in these patients at the time of the ileus were 244, 351, 444, and 554 ng/dL. In all patients, the ileus resolved within 1–2 weeks when the drug was withheld and did not reoccur when the drug was restarted at a lower dose. Two of the patients who did not respond to gossypol developed hypokalemia 2–3 weeks after discontinuation of gossypol. The serum potassium levels in these patients were 2.3 and 2.6 mmol/L (normal range, 3.5–4.0 mmol/L) and their urine free cortisol levels were 1850 and 11,800 nmol/day, respectively (normal 30–300 μmol/day). Ketoconozole was started at this time to decrease the cortisol secretion and the hypokalemia resolved with correction of the hypercortisolism.

## Discussion

The prognosis for patients with adrenal cancer is dismal. The majority of patients present with advanced disease at

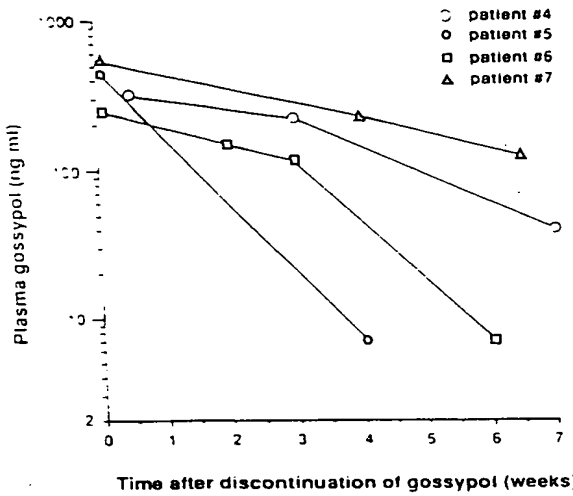


FIG. 4. Plasma gossypol concentrations after discontinuation of gossypol in four patients with metastatic adrenal cancer. The mean half-time of disappearance (±SD) was 2.9 ± 0.9 weeks (range, 2.5–4.0 weeks).

the time of diagnosis. Whereas surgical resection can prolong survival in some patients, most will eventually have inoperable disease and a 5-yr survival less than 20%. In these patients, medical therapy is generally disappointing. Mitotane can induce biochemical remissions in 60–70% of patients with hypercortisolism, but only 10–20% of patients have any objective decrease in tumor size (1, 9). The amount of tumor shrinkage with mitotane varies from 10–80% and these responses are generally short-lived, lasting 6–9 months. The side effects of mitotane include fatigue, anorexia, nausea, vomiting, ataxia, dizziness, confusion, and memory loss. Various conventional chemotherapeutic agents have also been used for adrenal cancer including cisplatin, 5-fluorouracil, cytoxan, adriamycin, vincristine, and VP-16. The partial response rates for these regimens are only 10–20% and the duration of response is 1 yr on average (2, 10–13). The side effects associated with these chemotherapeutic regimens include myelosuppression, nausea, vomiting, alopecia, and cardiotoxicity. Thus, a drug with greater efficacy and less toxicity would be desirable for the treatment of metastatic adrenal cancer.

Gossypol, a naturally occurring biphenolic compound derived from cottonseed, first came to attention as a cause of infertility in Chinese villages using crude cottonseed oil for cooking during times of economic deprivation. Subsequently, it has been shown to be a potent spermatotoxic agent and general antimetabolite (14–16). Gossypol has *in vitro* activity against several tumor cell lines derived from mouse mammary carcinoma, and human melanoma, colon, and adrenal carcinomas (3, 17). Tso and colleagues (4) demonstrated the *in vivo* antitumor activity of ip gossypol against Ehrlich ascites tumors in nude mice. The therapeutic dose range, however, was quite narrow and several animals died from the toxic effects of gossypol. Similarly, Rao and colleagues (5) demonstrated the activity of ip gossypol against mouse mammary tumors. Although 66% of the treated animals were tumor free, 34% died of drug toxicity. When we used oral gossypol, however, in nude mice bearing SW-13 human adrenal cancers, we did not observe any morbidity or mortality related to gossypol. On the contrary, we found that gossypol inhibited the growth of human adrenal tumors and enhanced the survival of the gossypol-treated mice compared to untreated controls (3). These observations led us to examine oral gossypol as a treatment for adrenal cancer in humans.

The largest studies using oral gossypol in humans are those from China where it has been studied extensively as a potential male contraceptive agent (6, 7, 15). In these studies, over 8000 normal men received oral gossypol at loading doses of 20 mg/day for several weeks followed by maintenance doses of 50–60 mg/week. The side effects were generally minor, with the only serious side effect being profound hypokalemia in less that 1% patients, with hypokalemic paralysis in a few patients. The paralysis was reversible, but required administration of iv potassium in some cases. Since idiopathic hypokalemia also occurs in the normal population in China, the role of gossypol in hypokalemia is unclear. Hypokalemia was not noted in a subsequent series of patients receiving gossypol in South America (18).

Based on these contraceptive trials, we initiated our trial of gossypol at a dose of 20 mg/day. Since there are no human studies using larger doses of gossypol, one of our objectives was to determine a safe dosage range. The maximum tolerated gossypol dose in our patients was 0.8 mg/kg day (50–60 mg/day). Doses above this were associated with excessive nausea, anorexia, and fatigue. None of our patients experienced hypokalemia directly related to gossypol. Two patients had hypokalemia after stopping gossypol, that was related to excessive cortisol secretion. None of the patients had to discontinue gossypol because of side effects. The only severe side effect was an abdominal ileus that occurred in four patients treated for an extended period of time at doses over 40 mg/day. This responded to withholding gossypol for 1–2 weeks and did not reoccur when gossypol was restarted at a lower dose.

The plasma gossypol levels in our patients correlated only roughly with the prescribed dose. There are several possible explanations for this finding. Since the half-life of gossypol is quite long, a true steady-state may not have been achieved in some patients leading to inconsistent plasma gossypol concentrations. In addition, the lipophilic nature of gossypol would cause it to accumulate in the body fat such that plasma gossypol levels would not reflect the body content of the drug. Finally, patient noncompliance may have been a factor as we were able to document at least one case where the prescribed dose was 40 mg/day and the patient was routinely taking only 30 mg/day.

Partial tumor responses were seen at gossypol doses of 0.6–0.8 mg/kg·day (40–60 mg/day). The plasma gossypol levels at the time of these tumor responses, however, were quite variable, ranging from 83–547 ng/dL. Our data do not give a clear indication of the minimum effective plasma gossypol concentration, because the plasma concentrations in patients who responded were indistinguishable from those in patients who did not respond. Again, the plasma concentrations may not accurately reflect the concentration of gossypol in the tumor. In our experience, higher doses of gossypol and higher plasma gossypol concentrations did not

correlate with increased tumor response. We are unable to fully explain why, in two patients who initially had tumor shrinkage, the plasma gossypol levels progressively declined despite continued prescription of gossypol at doses equal to or greater than those at the time of their response.

Overall, we observed a partial tumor response rate of 17% with a duration of response of several months to over 1 yr. The response rate with gossypol treatment was similar to that seen with the other chemotherapeutic agents available for adrenal cancer, consistent with the generally poor response of adrenal cancer to medical therapies. The tumor responses seen during gossypol treatment, however, occurred in patients who were refractory to other chemotherapeutic modalities. Thus, the response rate of gossypol should be viewed in the setting of a second line, salvage agent. As with other chemotherapeutic trials in adrenal cancer we were unable to include a placebo arm in our study. Thus, we cannot determine the effect of gossypol on patient survival. We also cannot definitely rule out spontaneous tumor necrosis as a factor in the tumor responses. The lack of spontaneous responses in historical series, however, and the timing of the responses in relation to gossypol administration, make this possibility unlikely (1, 9). There was no definite effect of gossypol on hormone synthesis. One patient who had a tumor response during gossypol treatment also had lower free cortisol excretion, but there were no hormonal responses in the other patients with secretory tumors. This suggests that the reduction in cortisol excretion seen during gossypol administration was due to decreased tumor mass, rather than a direct effect on the cortisol synthetic pathway.

The mechanism of action of gossypol in our patients with adrenal cancer is unknown. Recently, Benz and colleagues (19) studied the *in vitro* tumoricidal effects of gossypol on breast, ovarian, colon, and pancreatic cancer cell lines demonstrating selective destruction of mitochondria, accompanied by a decrease in intracellular ATP. Thus, gossypol may exert its cytotoxic effects by uncoupling oxidative phosphorylation. It is unclear, however, how this activity could specifically target tumor cells *in vivo*. Another potential mechanism of gossypol action stems from its ability to inhibit endothelial-derived relaxing factor (nitrous oxide), an agent responsible for blood vessel dilatation (20, 21). It is possible that, through this mechanism, gossypol interferes with tumor blood supply causing tumor necrosis. This could explain why one of our patients had nearly complete necrosis of a large abdominal lesion, but no effect on a small retroperitoneal lesion.

We conclude that oral gossypol can be used daily on an outpatient basis for the treatment of metastatic adrenal cancer. Although the partial response rate is low, it is comparable to the other medical therapies available, and it can be used as a salvage agent when other treatments for adrenal cancer have failed. This is the first indication that gossypol may have activity against cancer in humans and suggests the need for further studies to elucidate its mechanism of action and explore its role in the treatment of other human cancers.

## Acknowledgments

We are grateful to the clinical associates and nursing staff in the NIH Endocrine Unit and the New York Hospital-Cornell Medical Center Clinical Research Center for their excellent assistance with patient care.

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*Journal of Neuro-Oncology* 43: 79–86, 1999.


*Clinical Study*

# Gossypol treatment of recurrent adult malignant gliomas

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*Key words:* malignant glioma, gossypol, lactate dehydrogenase isoenzymes, chemotherapy

**Summary**

Gossypol, a polyphenolic compound which depletes cellular energy by inhibition of several intracellular dehydrogenases, has been shown to have antiproliferative activity against human glial tumor cell lines *in vitro* and in nude mouse xenografts. Human trials of gossypol as a male contraceptive have demonstrated safety of long-term administration. We studied the activity of Gossypol 10 mg PO bid in 27 patients with pathologically confirmed glial tumors which had recurred after radiation therapy. Fifteen patients had glioblastoma, 11 patients anaplastic astrocytoma, 1 patient relapsed low grade glioma. Response was assessed every 8 weeks using CT/MRI scan and clinical criteria including decadron requirement. Treatment was continued until disease progression. Two patients had partial response (PR); 4 had stable disease for 8 weeks or more. One patient maintained a PR with improved KPS for 78 weeks. The other had a PR lasting 8 weeks. Toxicity was mild: 2 heavily pretreated patients had mild thrombocytopenia, 5 patients developed hypokalemia, 3 patients developed grade 2 hepatic toxicity and peripheral edema. Gossypol levels measured by HPLC did not correlate with response or toxicity in this study.

We conclude that gossypol is well tolerated and has a low, but measurable, response rate in a heavily pretreated, poor-prognosis group of patients with recurrent glioma. The presumed novel mechanism of action, lack of significant myelosuppression, and activity in patients with advance glioma support further study of gossypol as an antineoplastic agent.

## Introduction

Glioblastomas and anaplastic astrocytomas are infiltrating tumors of the central nervous system which are rarely cured by surgery and radiation therapy. Glial malignancies seem to express intrinsic resistance to currently available chemotherapeutic agents and the addition of chemotherapy to the treatment of malignant gliomas either in the adjuvant setting or at time of relapse has only modestly improved the survival of patients compared with treatment with radiation therapy alone [1].

It has been recognized that malignant gliomas along with many other malignant neoplasms have alterations in their intermediary metabolism. Neoplastic tissues may rely on anaerobic metabolism and may utilize glycolysis and the pentose phosphate shunt as their major sources of energy metabolism [2,3]. Gliomas also have distinct lactate dehydrogenase (LDH) isoenzyme profiles compared to normal brain tissue. Specifically gliomas contain high levels of cationic forms of LDH ($LDH_4$ and $LDH_5$) [4–7]. These differences may allow the use of selective inhibition of tumor intermediary metabolism as a cytotoxic or cytostatic

treatment or as a sensitizing strategy for other treatment modalities.

Gossypol, a product in cottonseed oil, may be such a selective inhibitor of intermediary metabolism. The exact mechanisms of action of gossypol are not completely defined but it is known to accumulate within mitochondria and uncouple electron transport from oxidative phosphorylation [8]. This compound has been extensively studied as a possible male contraceptive agent, since it was found to inhibit metabolism in human sperm which uses cationic LDH (LDHX) as its source of energy [9]. Gossypol inhibits the activity of many enzymes including LDHX and other cationic forms of LDH thereby inhibiting glycolysis [10,11]. Other potentially relevant actions of gossypol have been demonstrated *in vitro* include inhibition of cell cycling by modulation of the regulatory proteins Rb and cyclin D1, elevation of TGF-beta 1 gene expression and inhibition of protein kinase C activity [12–14]. Although malignant transformation of astrocytic cells is multifactorial, all of the preceding alternations have been detected in glial tumors [15]. *In vitro* work showed that gossypol can modulate resistance to alkylating agents in conjunction with buthionine sulfoximine through inhibition of glutathione synthetase [16]. We have previously demonstrated that gossypol is cytotoxic to human glioblastoma cell lines *in vitro* and in nude mouse xenograft models [17]. Additional rationale for the use of gossypol in treatment of brain tumors is its long serum half life and high lipid solubility at physiologic pH which suggests that it should penetrate the blood–brain barrier [18].

Previous studies in humans have determined tolerability of oral administration: a phase II study of gossypol (30–70 mg/day orally for at least 6 weeks) in humans with adrenocortical cancer demonstrated 3 responses in 17 evaluable patients [19]. Gossypol has also been used in treatment of benign uterine myomatosis and ovarian carcinoma [20].

Based on these considerations, we undertook a phase II trial using continuous daily gossypol as a single agent in adult patients with recurrent or progressive high grade glioma. The goals of the study were: (1) To estimate the response rate to gossypol treatment. (2) To evaluate the progression free survival of patients who were treated with gossypol. (3) To assess the pharmacokinetics and toxicity of gossypol when used as an antineoplastic agent, especially since this will be the first clinical use in a population of patients treated concurrently with anticonvulsant medications.

## Gossypol assay

Gossypol acetic acid was obtained from the W Health Organization and its purity confirmed by H performance liquid chromatography (HPLC). St dards were made to contain 100, 200 and 400 ng gossypol in blank human plasma. Aliquots of 0.4 m each of the above standards and all samples in duplic were placed in 12 × 75 mm glass culture tubes to wh 10 µg/ml gossypol dimethyl ether solution in aceto trile were added as an internal standard. (Stock solut of gossypol acetic acid and gossypol dimethyl et were prepared in DMSO and stored frozen.) Prot was precipitated by addition of 0.4 ml acetonitrile each sample, vortexed, allowed to sit for 5 min at ro temperature and centrifuged at 3,000 rpm for 10 m The protein-free supernatant was transferred by Past pipet to new 16 × 125 mm glass extraction tubes w Teflon lined screw caps. To every tube, 2 ml of a s urated EDTA (disodium salt) solution maintained 37°C was added and vortexed. Organic material w extracted by adding 3 ml chloroform to each tube, rot ing gently (approximately 20 rpm on a mechanical ro tor) and then centrifuging for 15 min at 3,000 rpm. T organic material was transferred by Pasteur pipet t ing care to avoid any remaining aqueous material in 12 × 75 mm glass culture tubes in which 25 µl of a 1( (V/V) acetic acid/methanol solution had been evap rated to dryness. The organic material was evaporat to dryness under a stream of dry nitrogen in a wa (approx. 40°C) water bath. The residue was imme ately dissolved in 100 µl of a 20% water/80% aceto trite solution and then 4 µl analyzed by HPLC with electrochemical detector.

The HPLC system consisted of a Waters WISP 7 automatic injector, Waters 510 pumps and an E model 5100 Coulochem Detector with a model 50 Conditioning Cell and a model 5011 analytical ce All HPLC stainless steel tubing and fittings were pl placed with Upchurch's PEEK fittings to decrea background ions. The gossypol and internal standa were measured using a redox reaction. The settin were +0.65 V for the conditioning cell, +0.55 V detector 1 of the analytical cell and −0.35 V at dete tor 2 of the analytical cell. The gain was set at 30 × 1( (99 × 100 is the maximum setting). A Shodex H pak D18-613 stainless steel, 6 mm × 150 mm colun was used (this is a gel column bonded with C18). T mobile phase was 30% (V/V) 0.02 M phosphate buff pH 5.1: 70% acetonitrile at a flow rate of 1.5 ml/mi

The retention times were 6.8 min for gossypol and 9.4 min for gossypol dimethyl ether.

Recovery of gossypol from plasma was approximately 89%. The coefficient of variation for 10 replicates of 50 μg/ml gossypol was 5.67%. Over seven months the coefficient of variation was 12% for 400 ng/ml.

### Patients and treatment protocol

Patients were recruited for this study from two of the participants in the Buffalo-Rochester-Syracuse Neuro-Oncology Research Group: the SUNY Health Science Center in Syracuse and the University of Rochester Cancer Center. Patients were eligible for treatment if they had a histologically proven glioblastoma or anaplastic astrocytoma which had demonstrated progressive enlargement by CT or MRI scanning more than 8 weeks after debulking surgery and radiation therapy. Patients must have been 18 years of age or over, have acceptable bone marrow, renal and hepatic function, a Karnofsky performance status of greater than or equal to 60, and not be pregnant or lactating. The study was approved by the investigational review boards of all participating hospitals and patients signed written affirmation of informed consent prior to starting treatment. Diagnostic biopsies from all patients were reviewed by a pathologist.

Racemic gossypol acetic acid 10 mg compressed tablets were obtained from the Chinese Academy of Medical Sciences (Beijing, China). Patients were treated with a fixed dose of 10 mg orally twice a day taken 1 h prior to or 1 h after meals or antacids. The dose of 20 mg/day was chosen as the maximal tolerated dose for prolonged daily oral administration based on our experience with patients with adrenocortical carcinoma; doses of 30 mg/day or higher caused emesis in 13/16 patients [19,21]. Dexamethasone treatment was permitted during the study. Patients were encouraged to eat a diet rich in potassium and to avoid alcohol. Patients with serum potassium level less than 4.0 mEq/l were supplemented with KCL 20 mEq daily. Plasma was obtained for measuring gossypol level 4 and 12 weeks after initiating therapy and 4 weeks and 12 weeks after each dosage change. Gossypol was continued until the patient had evidence of progressive disease or developed greater than grade 2 toxicity. Patients were assessed for toxicity by physical and laboratory examination every 4 weeks, and by neuroradiologic examination every 8 weeks. Toxicities were graded according to standardized criteria previously reported by the ECOG [22]. Anticonvulsant drug levels were measured weekly while the patient was on study.

Response was defined by previously published clinical and radiographic findings [23]. All neuroradiologic studies were independently reviewed by a neurosurgeon and a neuroradiologist to confirm the results. In order to qualify for response assessment, the dose of dexamethasone must have remained stable or decreased.

### Results

A total of 27 patients were enrolled in this study. One patient was enrolled and took gossypol for three weeks, but withdrew consent for follow up so details of response and toxicity are not available. This patient was classified as having progressive disease since she was not known to benefit from treatment. The characteristics of the patients treated are summarized in Table 1. All had previous irradiation. Twenty six had prior chemotherapy, 11 with more than one regimen. Twenty two patients had received procarbazine containing regimens, most frequently MOP (mechlorethamine, vincristine and procarbazine), 10 had received BCNU.

All patients were assessed for toxicity. Three patients were taken off study drug before one could expect

*Table 1.* Characteristics of all patients treated with gossypol

| | |
|---|---|
| Total number of patients | 27 |
| Age | |
| Median | 49 |
| Range | 20–68 |
| Sex | |
| Male | 20 |
| Female | 7 |
| Histology | |
| Glioblastoma | 15 |
| Anaplastic astrocytoma or mixed anaplastic oligodendroglioma/astrocytoma | 11 |
| Recurrent low grade glioma with features on CT scan suggesting anaplastic transformation | 1 |
| Tumor Location | |
| Frontal | 9 |
| Temporal | 7 |
| Occipital or parietal | 6 |
| Central or thalamic | 5 |
| KPS score | |
| Median | 70 |

gossypol to have any anti-tumor effect: one on the third day of treatment when a deep venous thrombosis (DVT) was diagnosed, one had significant clinical deterioration after registration but prior to starting treatment and was taken off treatment after receiving only 3 days of gossypol, one had significant clinical deterioration consistent with progression 5 days after starting gossypol. One patient had stable tumor on imaging at eight weeks, but his dexamethasone dose had been increased prior to the scan so he was considered to have progressed by clinical criteria.

Response to therapy is summarized in Table 2. Two patients had PR. A 20-year-old male with glioblastoma involving the corpus callosum evidenced recurrence after 4 cycles of MOP chemotherapy and irradiation. He had a marked symptomatic and radiologic improvement 8 weeks after starting gossypol (Figure 1a), but by 16 weeks had clinical deterioration. The second patient was a 47-year-old female who had a right frontal glioblastoma which was resected and irradiated. She progressed on BCNU chemotherapy and underwent second partial resection which demonstrated persistent glioma. She continued to deteriorate neurologically, and postoperative CT scanning revealed increased enhancement and edema outside the surgical field, extending across the corpus callosum. Within 8 weeks of starting gossypol therapy the patient's neurological functioning improved and CT scans demonstrated a significant decrease in the degree of enhancement and mass effect (Figure 1b). This patient's response was sustained for 78 weeks. Overall, 19 patients progressed on gossypol treatment by 8 weeks or less, 6 patients progressed between 8 and 16 weeks of treatment, and two patients were treated for greater than 16 weeks. We found no correlation of time to progression with histology of the tumor.

Toxicities due to gossypol treatment are summarized in Table 3. Only 1 patient, the one diagnosed with a DVT on third day of treatment, stopped taking gossypol because of possible toxicity. All other patients were taken off treatment or expired due to progression of their tumor. One patient suffered an intracranial bleed without any other evidence of abnormal bleeding tendency or thrombocytopenia, felt to be unrelated to therapy, but due to necrosis of the progressing brain tumor.

Twenty three of 27 patients were treated with anticonvulsants concurrently with gossypol therapy: phenytoin 13, phenobarbital 9, carbamazepine 9, valproic acid 2 and felbamate 1 (11 patients were on more than one anticonvulsant). Although formal pharmacokinetic studies of the anticonvulsants were not performed, anticonvulsant levels were measured weekly and only 3 patients were recorded as requiring dosage changes during the study (increase phenytoin 1; decrease phenytoin 1; increase carbamazepine 1). From these observations we conclude that it is unlikely that gossypol significantly affects pharmacokinetics of commonly used anticonvulsants.

Plasma levels of gossypol were assessed 4–8 weeks after starting therapy in 10 patients. No difference was detected in mean plasma levels in responders: 115 ng/ml (3 pts assessed, range 60–217) and non-responders: 129 ng/ml in (7 pts, range 0–309).

*Table 2.* Summary of patient responses to gossypol

| Best response (27 patients) | No. of patients (%) | Histology of responding patients | Duration of response (weeks) |
|---|---|---|---|
| Partial response | 2 (7) | GBM 2 | 8, 78 |
| Stable disease | 4 (15) | GBM 2, AA 2 | 12, 12, 12, 13 |
| Progression | 21 (78) | | |

## Discussion

Despite the development of dozens of antineoplastic agents in the past three decades, the treatment of malignant glioma remains inadequate and the most active agents remain the DNA-active alkylating agents BCNU and procarbazine. The important differences in the intermediary energy metabolism of malignant gliomas compared to normal astrocytes represent an attractive target for new therapies which preferentially interfere with the metabolism of tumor cells. This study examined the activity of such an agent, gossypol, in the treatment of recurrent glial tumors.

The exact mechanism of action of gossypol is not determined. It is a highly reactive compound shown to interact with many intracellular enzymes and regulatory proteins *in vitro* [12–14,24–26]. We studied the effects of gossypol on CNS tumor cell lines and patients with brain tumors based on findings of inhibition of cationic forms of LDH, which are isoenzymes reported to be overexpressed in glial tumors [17].

It is important to consider the toxicities of proposed agents for the treatment of glial tumors. Patients who have received nitrosourea chemotherapy frequently have diminished bone marrow and pulmonary reserve and may be at higher risk of adverse reactions from intensive chemotherapy. Patients with space occupying

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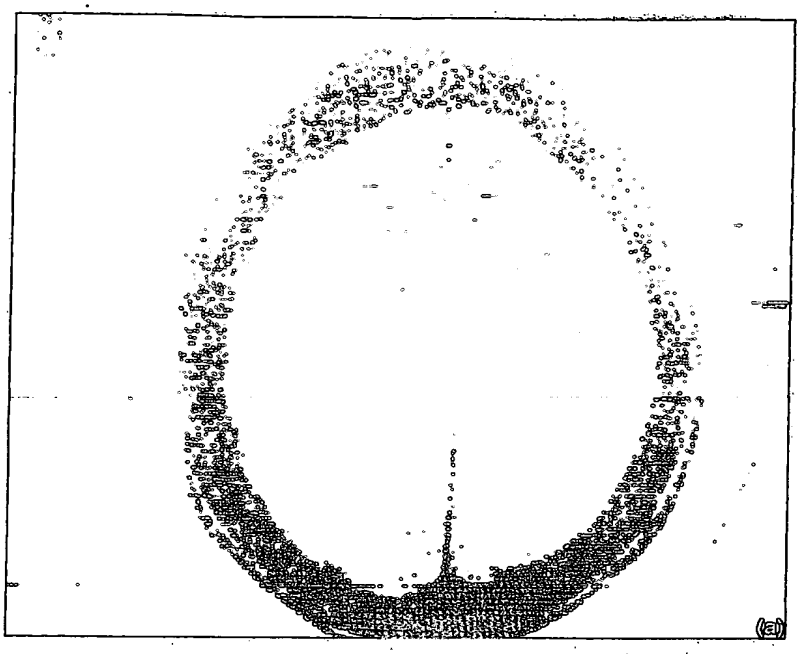


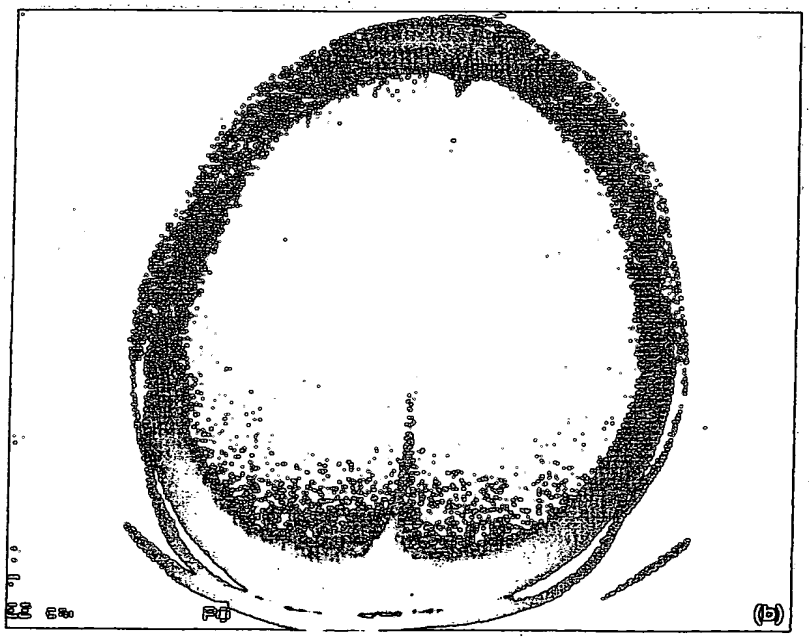


*Figure 1a,b.*

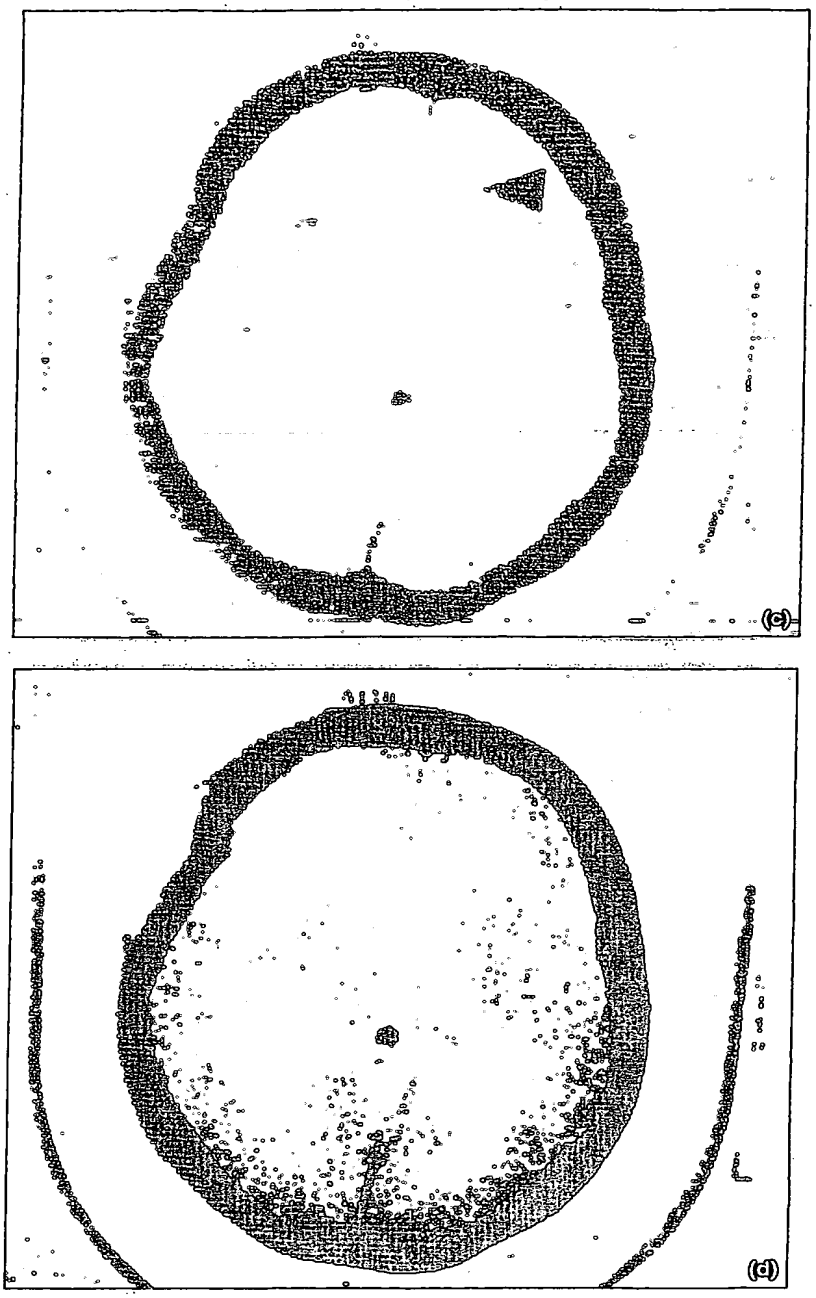

*Figure 1.* (a) Twenty-year-old male with glioblastoma of corpus callosum recurrent after irradiation and MOP chemotherapy. (b). Partial response after 8 weeks of therapy with gossypol. (c) Forty-seven-year-old female with right frontal glioblastoma recurrent after irradiation, BCNU chemotherapy and second debulking surgery. Arrowhead indicates edema and mass effect in contralateral frontal hemisphere. (d) Partial response after 8 weeks of therapy with gossypol.

Summary of patient toxicities to gossypol

| Toxicities | NCI common toxicity criteria grade | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Hepatic | 6 | 3 | | | |
| Leukopenia | 1 | | | | |
| Thrombocytopenia | 2 | | | | |
| Hypokalemia | 7 | 1 | 1 | | |
| Gastrointestinal* | 1 | | | | |
| Cardiac | | | | | |
| Renal | | | | | |
| Pulmonary | | | | | |
| Xerostomia | 1 | | | | |
| Xeropthalmia | 1 | | | | |

*One patient complained of grade 1 nausea throughout gossypol therapy, 1 patient had mild abdominal pain during therapy, which resolved with cessation of therapy, 1 patient had an ileus at the time of neurologic progression felt to be possibly related to therapy and 1 patient with small bowel obstruction 3 weeks after stopping therapy, felt to be unrelated to therapy.

intracranial lesions may also have a high incidence of seizures and be sensitive to changes in intracranial pressure which may affect their ability to tolerate therapy. Our experience was that gossypol was well tolerated in this group of debilitated patients. Gossypol may be an attractive agent for further study since it had mild toxicities, and no recognizable effect on anticonvulsant levels in the patients treated.

There is a literature on the toxicity of gossypol in animals and in humans. Gossypol is a contaminant in cotton seed and was present in livestock feeds containing cotton seed meal. It was also a contaminant of raw cotton seed oil in a part of China. The gossypol caused azoospermia which led to Chinese research on gossypol as a potential oral contraceptive for men.

Adverse events associated with gossypol in clinical studies at 60–70 mg/day include change in appetite, fatigue, dry mouth, diarrhea, and transaminase elevation [9]. At doses of 20 mg/day, the effects were less and included weakness, decrease in appetite, increase in appetite, dry mouth and nausea. A number of subjects in the south of China also developed hypokalemia [9] but some healthy men not taking any medicine and living in Shanghai are hypokalemic by conventional standards (serum potassium concentration less than 3.5 mM) [27,28]. Loss of sperm motility followed by decline in sperm count also occurred in all men taking adequate dose of gossypol for an adequate duration of time. Ovarian suppression and endometrial atrophy have also been described [29].

In two small studies in cancer patients, anorexia, nausea and vomiting were the main dose-limiting toxicities with an apparent paralytic ileus occurring after 3–4 months of therapy in some patients [19,21].

The major endpoint of this study was response rate. Interpretation of the response of recurrent glial tumors to therapy can be problematic [30]. We stopped our study based on our finding of a low response rate in this poor-prognosis, unselected group of patients. The objective response rate was low but 2 heavily pretreated patients had definite partial responses. Radiologically documented decrease in area of enhancement is considered by many to be the best evidence of cytotoxic activity of the agent being studied, but lack of progression of what are often highly malignant tumors can also be a valuable clinical endpoint. The response rate at this dose and schedule of gossypol in relapsed patients does not appear to be high enough to warrant the use of this agent as primary treatment in unselected patients. However, considering that steady state plasma levels of gossypol are not attained for at least 8 weeks when the drug is administered orally, whereas patients with recurrent high grade glioma often have rapid progression and clinical deterioration our findings suggest that further clinical trials should be designed to incorporate gossypol at an earlier clinical stage of this disease. Further studies to determine and predict biologic responsiveness to gossypol may be feasible once the mechanism of action of this agent is better understood, thus allowing for better patient selection. We think that our experience justifies further study of gossypol as an antineoplastic medication and further research using gossypol as the lead compound to discover a potentially novel class of antineoplastic drugs.

## Acknowledgements

This study was supported in part by funding from the Buffalo-Rochester Syracuse Neuro-Oncology Research Group Inc. We thank Sharon Longo for technical support.

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Patent Application No. 10/806,088

Applicant: Flack et al.

Filed: March 22, 2004

TC/AU: 1614

Examiner: Weddington, Kevin E.

Docket No.: 225011 (Client Reference No. E-133-1990/0-US-03)

Customer No.: 45733

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Alexandria, VA 22313-1450

## INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 CFR 1.97 and 1.98, the references listed on the enclosed Form PTO-1449 and/or Substitute Form PTO-1449 ("Form 1449") are submitted for consideration by the Examiner in the examination of the above-identified patent application.

The full consideration of the references in their entirety by the Examiner is respectfully requested and encouraged. Also, it is respectfully requested that the references be entered into the record of the present application and that the Examiner place his or her initials in the appropriate area on the enclosed Form 1449, thereby indicating the Examiner's consideration of each of the references.

The submission of the references listed on the Form 1449 is for the purpose of providing a complete record and is not a concession that the references listed thereon are prior art to the invention claimed in the patent application. The right is expressly reserved to establish an invention date earlier than the above-identified filing date in order to remove any reference submitted herewith as prior art should it be deemed appropriate to do so.

Further, the submission of the references is not to be taken as a concession that any reference represents art that is relevant or analogous to the claimed invention. Accordingly, the right to argue that any reference is not properly within the scope of prior art relevant to an examination of the claims in the above-identified application is also expressly reserved.

The Information Disclosure Statement is being filed:

☐ **within** any one of the following time periods: (a) within three months of the filing date of a national application other than a continued prosecution application under 37 CFR 1.53(d); (b) within three months of the date of entry of the national stage as set forth in 37 CFR 1.491 of an international application; (c) before the mailing date

of a first Office Action on the merits; or (d) before the mailing of a first Office Action after the filing of a request for continued examination under 37 CFR 1.114.

☒ **after** (a), (b), (c) or (d) above, but before the mailing date of a final action under 37 CFR 1.113, a Notice of Allowance under 37 CFR 1.311, or an action that otherwise closes prosecution in the application, and includes *one* of:

- ☐ the Statement under 37 CFR 1.97(e) (see "Statement under 37 CFR 1.97(e)" below).

*or*

- ☒ the fee of $180 set forth in 37 CFR 1.17(p) (see "Fees" below).

☐ **after** the mailing date of a final action under 37 CFR 1.113 or a Notice of Allowance under 37 CFR 1.311, or an action that otherwise closes prosecution in the application, and on or before payment of the issue fee, and includes the Statement under 37 CFR 1.97(e) (see "Statement under 37 CFR 1.97(e)" below), and the fee of $180 as set forth in 37 CFR 1.17(p) (see "Fees" below).

☐ **after** the mailing date of a Notice of Allowance under 37 CFR 1.311, and on or before payment of the issue fee, and **within** thirty days of receiving each item of information contained in the Information Disclosure Statement, and includes the Statement under 37 CFR 1.704(d) (see "Statement under 37 CFR 1.704(d)" below), and the fee of $180 as set forth in 37 CFR 1.17(p) (see "Fees" below).
NOTE: This is for original applications except applications for a design patent, filed on or after May 29, 2000, wherein a paper containing only an Information Disclosure Statement in compliance with 37 CFR 1.97 and 1.98 is being filed.

**Copies of the References**

☐ Copies of all of the references listed on the enclosed Form 1449 are enclosed herewith.

☒ Copies of U.S. patents and patent applications that are listed on the accompanying Form 1449 are not enclosed herewith. Copies of other references identified on the accompanying Form 1449 are enclosed herewith.

☐ Attached to each reference not in the English language is a concise explanation of the relevance pursuant to 37 CFR 1.98(a)(3). An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of the relevance pursuant to 37 CFR 1.98(a)(3).

☐ A copy of the foreign search report is enclosed herewith.

☐ The references listed on the enclosed Form 1449 were previously identified in the parent application(s) of the present application, and copies of the references were furnished at that time. Accordingly, additional copies of the references are not

submitted herewith, so as not to burden the file with duplicate copies of references. The Examiner is respectfully requested to carefully review the references in accordance with the requirements set out in the Manual of Patent Examining Procedure. In accordance with 37 CFR 1.98(d), the details of the parent application(s) relied upon for an earlier filing date under 35 USC 120 in which copies of the references were previously furnished are set out below:

| U.S. APPLICATIONS | | STATUS *(check one)* | | |
|---|---|---|---|---|
| U.S. APPLICATIONS | U.S. FILING DATE | PATENTED | PENDING | ABANDONED |
| 1. | | | | |
| 2. | | | | |
| 3. | | | | |

**Statement under 37 CFR 1.97(e)**

☐ The **undersigned** hereby states that each item of information contained in the Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign patent application not more than three months prior to the filing of the Information Disclosure Statement.

☐ The **undersigned** hereby states that no item of information contained in the Information Disclosure Statement was cited in a communication from a foreign patent office in a counterpart foreign patent application, and, to the knowledge of the undersigned after making reasonable inquiry, no item of information contained in the Information Disclosure Statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the Information Disclosure Statement.

**Statement under 37 CFR 1.704(d)**

☐ The **undersigned** hereby states that each item of information contained in the Information Disclosure Statement was cited in a communication from a foreign patent office in a counterpart application and that this communication was not received by any individual designated in 37 CFR 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement.

**Fees**

☐ **No fee** is owed by the applicant(s).
☒ Charge Deposit Account No. 12-1216 in the amount of **$180.00** (37 CFR 1.17(p)). (A duplicate copy of this communication is enclosed for that purpose.)

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